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"If we knew what it was we were doing,

it would not be called research, would it?"

Albert Einstein

Acknowledgments

The PhD project is not something you simply do by individually. Rather, it is a journey that takes many years to complete, and is full of great challenges and enormous frustrations. During this journey the traveler is dependent on many people. In my case, this journey was accompanied by an excellent group of people who supported me in various ways to ultimately bring this journey to a successful end. Therefore I would like to thank them all for their competences, and guiding me throughout the journey.

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Summary

Isoprenoids comprise an extremely large and diverse group of natural compounds with more than 40,000 described examples. Isoprenoids are involved in numerous biological and ecological roles in the native organisms. From an industrial point of view, isoprenoids are becoming industrially important compounds because of their potent applications as anticancer, antitumor, antiviral, antibiotic, nutraceuticals, feed supplements as well as their characteristic flavors and aromas. Although the current large-scale processes for the production of isoprenoids rely mainly on extraction from plant sources or chemical synthesis these methods in many cases cannot support a viable industrial production. Heterologous microbial production of isoprenoids is therefore considered as a suitable alternative to extraction from plants or chemical synthesis.

The aim of this PhD study was to establish the yeast *Saccharomyces cerevisiae* as a platform for production of sesquiterpenes (a group of isoprenoids). *S. cerevisiae* exhibits several advantages as an isoprenoid producer platform including availability of its genome sequence, well established techniques required for cultivation and genetic manipulation, and its public acceptance as a GRAS (Generally Regarded As Safe) organism. Expression of three different plant sesquiterpene genes namely valencene synthase, cubebol synthase and patchoulol synthase resulted in the accumulation of the corresponding sesquiterpenes in the medium culture. However, a substantial amount of excreted sesquiterpenes were lost through the off-gas. Therefore a two-phase fermentation using dodecane as the secondary phase was developed for characterization of the engineered sesquiterpene producing yeast strains in batch fermentations.

In the next step, the mevalonate pathway of yeast strains was deregulated to augment the intracellular pool of the farnesyl diphosphate (FPP) which is the precursor for sesquiterpene biosynthesis. Deregulation of the yeast mevalonate pathway was performed through altered

expression of two main regulatory genes in the pathway namely *ERG9* and *HMG1*. Attenuated expression of *ERG9* by replacing the native *ERG9* promoter with a regulatable *MET3* promoter in the presence of methionine led to reduced ergosterol content of cells, accumulation of sesquiterpenes and also formation of farnesol as an FPP derived by-product. Overproduction of the catalytic domain of HMG-CoA reductase encoded by *tHMG1* also resulted in accumulation of squalene and higher titer of cubebol.

In a further attempt, *in silico* metabolic engineering approach was used to identify new target genes whose deletions could improve sesquiterpene biosynthesis in yeast. Deletion of NADPH-dependent glutamate dehydrogenase encoded by *GDH1* identified by this method led to significantly higher cubebol production.

Finally, heterologous production of lycopene in *S. cerevisiae* by expression of codon optimized *crtE*, *crtB*, and *crtI* genes from the bacterium *Erwinia herbicola* was investigated. Expression of the three carotenogenic genes led to accumulation of lycopene in the yeast strains. The effects of *ERG9* down-regulation and *tHMG1* overexpression on lycopene accumulation were also examined.

Dansk Sammenfatning

Isoprenoider er navnet på en kemisk stofgruppe og består af en diverse samling af naturligt forekomne forbindelser. De udfylder en lang række af biologiske og økologiske funktioner, og mere end 40,000 eksempler er beskrevet i litteraturen. Industrielt er isoprenioder i stigende grad blevet en interessent stofgruppe grundet deres virksomme egenskaber i forbindelse med behandling af kræft og virusinfektioner, men også i forbindelse med helseprodukter, og som smags og duft tilsætningsmiddel har isoprenoider fundet anvendelse. Den traditionelle fremstilling af isoprenoider har hidtil primært været baseret på ekstraktion fra planter eller gennem kemisk synthese, men disse metoder er resource krævene og problematiske set fra en økonomisk synsvinkel. Produktion af isoprenoider med hjælp fra rekombinante mikroorganismer synes derfor som et godt alternativ til de traditionelle metoder. Målet med dette Ph.D. studie har været at etablere en produktionsplatform i bagegær, *Saccharomyces cerevisiae*, for en undergruppe af Isoprenioder kaldet sesquiterpener. Valget af *S. cerevisiae*, som produktionsplatform, er udover kendskab til den fulde genomsekvens, baseret på en lang række af de fordele som knytter sig til denne organisme, eksempelvis et godt kendskab til dyrkningsmetoder og teknikker til genetisk modifikation samt den generelle accept af bagegær som en uskadelig organisme (GRAS). Ekspression af tre forskellige sesquiterpen gener fra planter, valencene synthase, cubebol synthase samt pathchoulol synthase, resulterede i akkumulering af de forventede sesquiterpener i vækstmediet, men det blev samtidig konstateret at en stor del af disse forbindelser grundet deres flygtige egenskaber blev tabt via afgangsgassen under dyrkningsprocessen. Dette dannede grundlag for udviklingen af en to-fase gæringsproces hvori dodecane bliver anvendt som anden fase, og denne teknik blev efterfølgende anvendt til

karakterisering af de udviklede sesquiterpene producerende gærstammer under de i projektet udførte gæringsforsøg. I det næste udviklingstrin blev regulering af mevalonate stofskiftet afkoblet i et forsøg på at ændre den intercellulære koncentration af farnesyl diphosphat (FPP), der udgør udgangspunktet for sesquiterpene biosynthesen. Denne afkobling blev gennemført ved ændring af udtrykket af to af de mest betydningsfulde regulatoriske gener i mevalonate stofskiftet, kaldet *ERG9* og *HMG1*. For at opnå reduceret udtryk af genet *ERG9* blev den oprindelige promoter udskiftet med den svagere og inducerbare *MET3* promoter, og i nærvær af aminosyren methionin blev et reduceret indhold af stoffet ergosterol målt i cellerne. Desuden blev der målt akkumulering af sesquiterpener og farnesol som er et biproduct af FPP. Overudtryk af det katalytiske domæne af HMG-CoA reduktase, der kodes af genet *tHMG1*, resulterede også i akkumulering af squalener og højere koncentrationer af cubebol.

Videre undersøgelser baseret på anvendelse af *in silico* metoder (metabolic engineering) identicerede to nye gener, hvis fjernelse kunne forbedre biosyntesen af sesquiterpener i gær. Fjernelsen af genet *GDH1*, der koder for det NAPDH-afhængige enzym glutamat dehydrogenase, der blev identificeret via den tidligere beskrevne fremgangsmåde, resulterede i et betydeligt højere udbytte af cubebol.

Afslutningsvis blev rekombinant produktion af lycopene i *S. cerevisiae* undersøgt gennem udtryk af de kodon-optimerede gener *crtE*, *crtB* og *crtI* fra bakterien *Erwinia herbicola*, og udtryk af de tre karoten-gener medførte akkumulering af lycopener. Effekten af nedregulering af *ERG9* og overudtryk af *tHMG1* i forbindelse med produktionen af lycopene blev ligeledes undersøgt.

Outline of the thesis

The current thesis is divided into the following sections:

Chapter 1 introduces isoprenoids, the metabolic pathways for the biosynthesis of isoprenoid precursors in different organisms, genes involved in each pathway, regulation of pathways, and a summary of metabolic engineering studies for the heterologous production of various isoprenoids in microbial hosts. This chapter is mainly based on the publication: Maury J, Asadollahi MA, Møller K, Clark A, Nielsen J. 2005. Microbial isoprenoid production: An example of green chemistry through metabolic engineering. Adv Biochem Eng Biotechnol 100:19-51.

Chapter 2 is dedicated to the development of a two-phase fermentation strategy for *in situ* separation of sesquiterpenes. The developed two-phase fermentation was further used for characterization of all sesquiterpene producing strains in batch fermentations throughout the project.

Chapter 3 deals with the effect of *ERG9* down-regulation on the ergosterol content of yeast cells, improvement of the intracellular pool of FPP and enhanced production of sesquiterpenes. A number of yeast strains producing valencene, cubebol or patchoulol were characterized in batch fermenters and effect of *ERG9* down-regulation, promoter and also carbon source on sesquiterpene production was studied. The results from this chapter were based on the publication: Asadollahi MA, Maury J, Møller K, Nielsen KF, Schalk M, Clark A, Nielsen J.

2008. Production of plant sesquiterpenes in *Saccharomyces cerevisiae*: Effect of *ERG9* repression on sesquiterpene biosynthesis. Biotechnol Bioeng 99:666-677.

Chapter 4 demonstrates how the flux towards FPP can be increased by overproduction of the catalytic domain of HMG-CoA reductase (encoded by *tHMG1*) which catalyzes the main flux controlling step in the mevalonate pathway. Influence of overexpression of *tHMG1* and its combination with *ERG9* down-regulation on cubebol biosynthesis and squalene and ergosterol content of yeast cells is discussed.

Chapter 5 illustrates how *in silico* metabolic engineering using the genome-scale reconstructed metabolic network of *S. cerevisiae* can be used for the identification of new target genes to improve sesquiterpene production in the yeast strains. Manipulation of the ammonium assimilation pathway by deletion of NADPH-dependent glutamate dehydrogenase encoded by *GDH1* that was identified by *in silico* metabolic engineering approach enhanced significantly cubebol production. Overexpression of NADH-dependent glutamate dehydrogenase encoded by *GDH2* restored the assimilation of ammonium and the specific growth rate of the yeast strains.

Chapter 6 explains construction of lycopene accumulating yeast strains by expression of *crtE*, *crtB*, and *crtI* genes from the bacterium *E. herbicola*. Expression of these genes in yeast led to accumulation of lycopene and appearance of a reddish color in the yeast strains. Effects of *tHMG1* overexpression and *ERG9* down-regulation on lycopene accumulation were studied. All strains were characterized in 5 L batch fermenters.

Chapter 7 concludes the thesis by summarizing the obtained results. This chapter also proposes strategies for further improvement of isoprenoid producing yeast strains.

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Chapter 2

Chapter 3

Appendix A

Abbreviations

ADS	Amorphadiene synthase
ATP	Adenosine triphosphate
CDP-ME	4-diphosphocytidyl-2-C-methyl-D-erythritol
CDP-ME2P	2-phospho-4-diphosphocytidyl-2-C-methyl-D-erythritol
CMP	Cytidine monophosphate
CoA	Coenzyme A
CTP	Cytidine triphosphate
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
DW	Dry weight
DXP	1-deoxy-D-xylulose 5-phosphate
ERAD	Endoplasmic reticulum associated degradation
FBA	Flux balance analysis
5-FOA	5-fluoroorotic acid
FPP	Farnesyl diphosphate
GAP	D-glyceraldehyde 3-phosphate
GC	Gas chromatography
GGPP	Geranylgeranyl diphosphate
GPP	Geranyl diphosphate
gTME	Global transcription machinery engineering

HMBPP	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HPLC	High-performance liquid chromatography
IPP	Isopentenyl diphosphate
LLE	Liquid-liquid extraction
MECDP	2-C-methyl-D-erythritol 2,4-cyclodiphosphate
MEP	2-C-methyl-D-erythritol 4-phosphate
MOMA	Minimization of metabolic adjustment
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PCR	Polymerase chain reaction
PDMS	Polydimethyl siloxane
PE	Perkin Elmer
PEP	Phosphoenolpyruvate
RFP	Red fluorescent protein
RNA	Ribonucleic acid
SIM	Selected ion monitoring
SPME	Solid phase microextraction
TIGRs	Tunable intergenic regions
TPP	Thiamine diphosphate
tRNA	Transfer ribonucleic acid
WT	Wild type
YPD	Yeast peptone dextrose

Chapter 1
Introduction

1.1 Isoprenoids

Isoprenoids (also referred to as terpenoids) are a diverse group of natural compounds with more than 40,000 identified compounds (Withers and Keasling, 2007); most of them are found in plants as constituents of essential oils (McCaskill and Croteau, 1997). Isoprenoids are derived from five-carbon isoprene units (2-methyl-1,3-butadiene) and the combination of isoprene units leads to the formation of different isoprenoids. Based on the 'isoprene rule' that was first recognized in 1887 by Wallach (1887) and later, in 1953, was extended to the 'biogenetic isoprene rule' by Ruzicka (1953), isoprenoids can be divided into different groups depending on the number of isoprene units in their carbon skeleton (Table 1.1).

Table 1.1. Classification of isoprenoids based on the number of isoprene units

Class	Isoprene units	Carbon atoms	Formula
Monoterpenoids	2	10	$C_{10}H_{16}$
Sesquiterpenoids	3	15	$C_{15}H_{24}$
Diterpenoids	4	20	$C_{20}H_{32}$
Sesterterpenoids	5	25	$C_{25}H_{40}$
Triterpenoids	6	30	$C_{30}H_{48}$
Tetraterpenoids	8	40	$C_{40}H_{64}$
Polyterpenoids	> 8	> 40	$(C_5H_8)_n$

The universal biological precursor for all isoprenoids is isopentenyl diphosphate (IPP) (Figure 1.1). Since the 1960s, when Bloch and Lynen discovered the mevalonate pathway for cholesterol synthesis (Katsuki and Bloch, 1967; Lynen, 1967) and until recently, IPP was assumed to be synthesized through the mevalonate-dependent pathway in all living organisms. However, in the 1990s, the existence of an alternative pathway, called the 2-methylerythritol 4-phosphate (MEP) pathway, was demonstrated in bacteria, green algae, and higher plants (Rohmer et al., 1993; Rohmer, 1999; Broers, 1994; Schwarz, 1994).

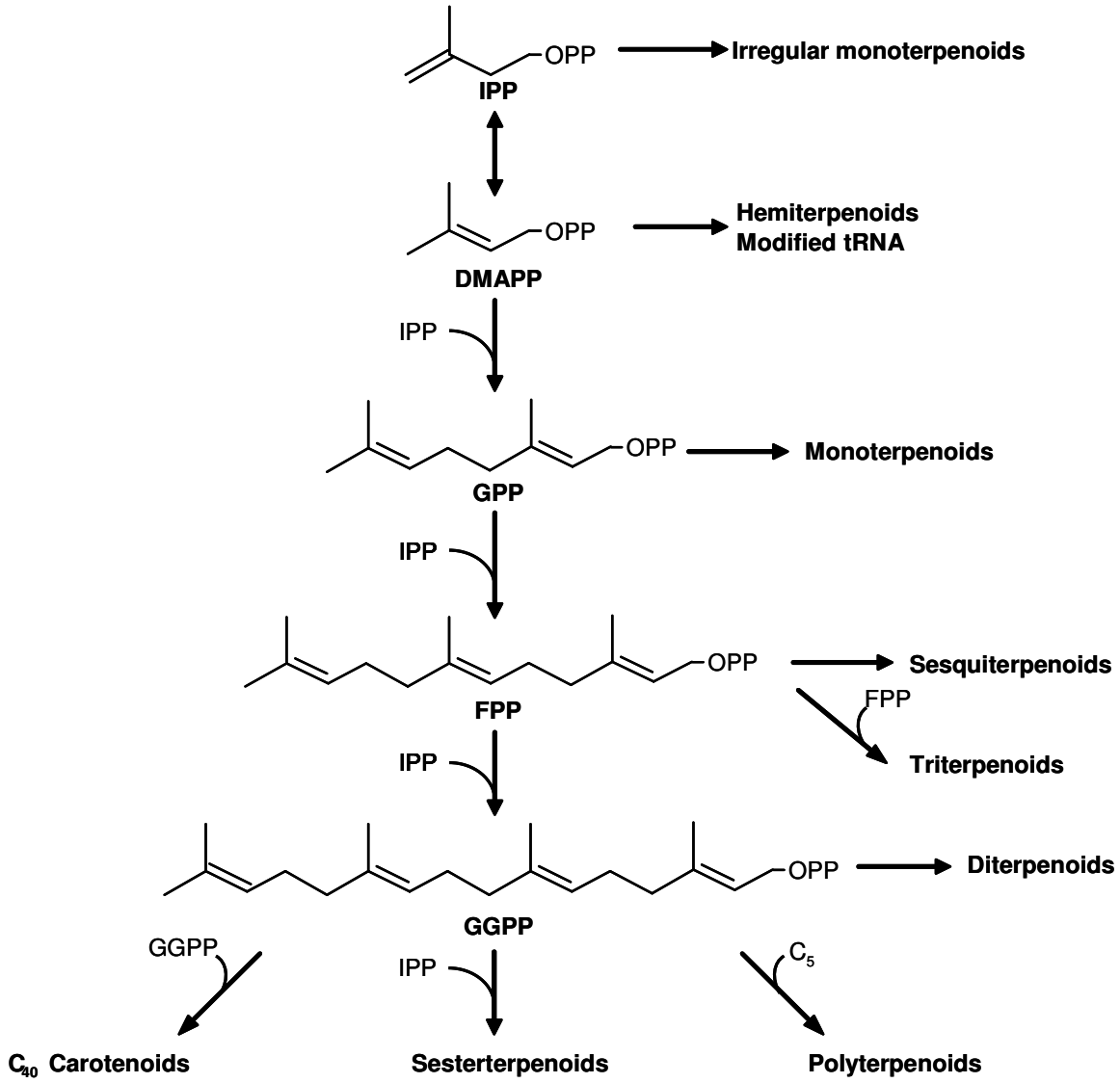

Figure 1.1. The different classes of isoprenoids and their precursors
DMAPP: dimethylallyl diphosphate, IPP: isopentenyl diphosphate, GPP: geranyl diphosphate, FPP: farnesyl diphosphate, GGPP: geranylgeranyl diphosphate.

Isoprenoids are functionally important in many different parts of cell metabolism such as photosynthesis (carotenoids, chlorophylls, plastoquinone), respiration (ubiquinone), hormonal regulation of metabolism (sterols), regulation of growth and development (giberellic acid, abscisic acid, brassinosteroids, cytokinins, prenylated proteins), defense against pathogen attack, intracellular signal transduction (Ras proteins), vesicular transport within the cell (Rab proteins) as well as defining membrane structures (sterols, dolichols, carotenoids) (Sacchettini and Poulter, 1997; Bach et al., 1999). Many isoprenoids also have considerable medical and commercial interest as flavors, fragrances (e.g. limonene, menthol, camphor), food colorants (carotenoids) or pharmaceuticals (e.g. bisabolol, artemisinin, lycopene, Taxol). In Table 1.2, some examples of isoprenoids and their corresponding biological functions or commercial applications are listed.

Isoprenoids are widely present in plant tissues and extraction from plants has been the traditional option for the large-scale production of these compounds. However, in many cases this method is neither feasible nor economical. Among the drawbacks in using plants as a source for isoprenoid production are influence of geographical location and weather on the composition and concentration of isoprenoids in the plant tissues, low concentration and poor yields for recovery of isoprenoids from plants and high costs associated with extraction and purification. Taxol, known as the most important anti-cancer drug introduced in the last fifteen years (Kingston, 2001), was originally extracted from the bark of the Pacific yew (*Taxus brevifolia*) through an extensive isolation and purification process. However, extraction of enough Taxol to treat one cancer patient requires six 100-year-old Pacific yew tree (Horwitz, 1994).

Chemical synthesis of isoprenoids has also been reported (Mukaiyama et al., 1999; Danishefsky et al., 1996; Miyaoka et al., 2002) and currently most of the industrially interesting carotenoids are produced via chemical synthesis (Sandmann et al., 1999). Nevertheless, because of the complex structure of isoprenoids, chemical synthesis, involving many steps, is difficult. Side reactions, unwanted side products, and low yield are other disadvantages. *In vitro* enzymatic production of isoprenoids through the act of plant isoprenoid synthases is also not practical because of dependency on the expensive precursors as well as poor *in vitro* conversion.

Microbial production of chemicals is an accepted environmentally friendly method that may lead to the production of large amounts of high-value isoprenoids from simple and cheap carbon sources. Engineered microorganisms would also enable production of unusual and novel isoprenoids with excellent biological and commercial applications.

Table 1.2. Biological activities or commercial applications of typical isoprenoids

Class	Biological activities[a]	Commercial applications[a]	Examples
Monoterpenoids	Signal molecules, e.g. as defense mechanism against pathogens	Flavors, fragrances, cleaning products, anticancer agents, antimicrobial agents	Limonene, menthol, camphor
Sesquiterpenoids	Antibiotic, antitumor, antiviral, immunosuppressive, and hormonal activities	Flavors, fragrances, potential pharmaceuticals	Juvenile hormone, nootkatone, artemisinin
Diterpenoids	Hormonal activities, antitumor properties	Anticancer agents	Gibberellins, phytol, Taxol
Sesterterpenoids	Cytostatic activities	None as yet	Haslenes
Triterpenoids	Membrane components	Biological markers	Sterols, hopanoids
Tetraterpenoids	Antioxidants, photosynthetic components, pigments, and nutritional elements	Food additives (colorants, antioxidants), anticancer agents	Lycopene, β-carotene
Polyterpenoids	N-linked protein glycosylation, side chains of ubiquinones	Rubber	Dolichols, prenols/quinones rubber

[a] Biological functions and commercial applications are selected examples.

Directed manipulation of cell factories using genetic engineering techniques requires detailed information about the metabolic pathways and enzymes involved in the biosynthesis of the desired product(s) and also an understanding of the mechanisms by which the flux through the pathway is controlled. One of the major obstacles for the commercial production of isoprenoids by cell factories is the limited supply of precursors. Replenishing the intracellular pool of precursors will need deregulation of pathways in order to improve the flux towards the biosynthesis of isoprenoid precursors. Therefore, before dealing with the investigations conducted in order to construct enhanced strains capable of isoprenoid production, the metabolic pathways for isoprenoid biosynthesis, their enzymes and genes and also the regulatory network of pathways are discussed.

1.2 The mevalonate pathway of *Saccharomyces cerevisiae*

Due to the involvement of isoprenoids in a variety of physiologically and medically important processes, the sterol biosynthetic pathway or mevalonate pathway has been intensively studied in eukaryotes. Principal end products of the mevalonate pathway are sterols, e.g. cholesterol in animal cells and ergosterol in fungi, which are important regulators of membrane permeability and fluidity (Daum et al., 1998; Veen and Lang, 2004). In addition to sterols, the mevalonate pathway provides intermediates for the synthesis of a number of other essential cellular constituents like hemes, quinones, dolichols or isoprenylated proteins which are all derived from the early part of the pathway (Lees et al., 1999). Thus, the mevalonate pathway can be considered to consist of two distinct parts: an early isoprenoid portion of the pathway, common to many branches and ending with the formation of farnesyl diphosphate (FPP), and a late part of the pathway mainly dedicated to ergosterol biosynthesis in *S. cerevisiae* (Figure 1.2). This partition of the pathway is also reflected by the oxygen requirement of some enzymatic steps in the second part of the pathway while this constraint does not exist for the first part of the pathway (Figure 1.2). As the early steps of the mevalonate pathway generate precursors for isoprenoid production, the next paragraphs will focus on the enzymes catalyzing these steps with emphasis on the key regulatory points of the pathway.

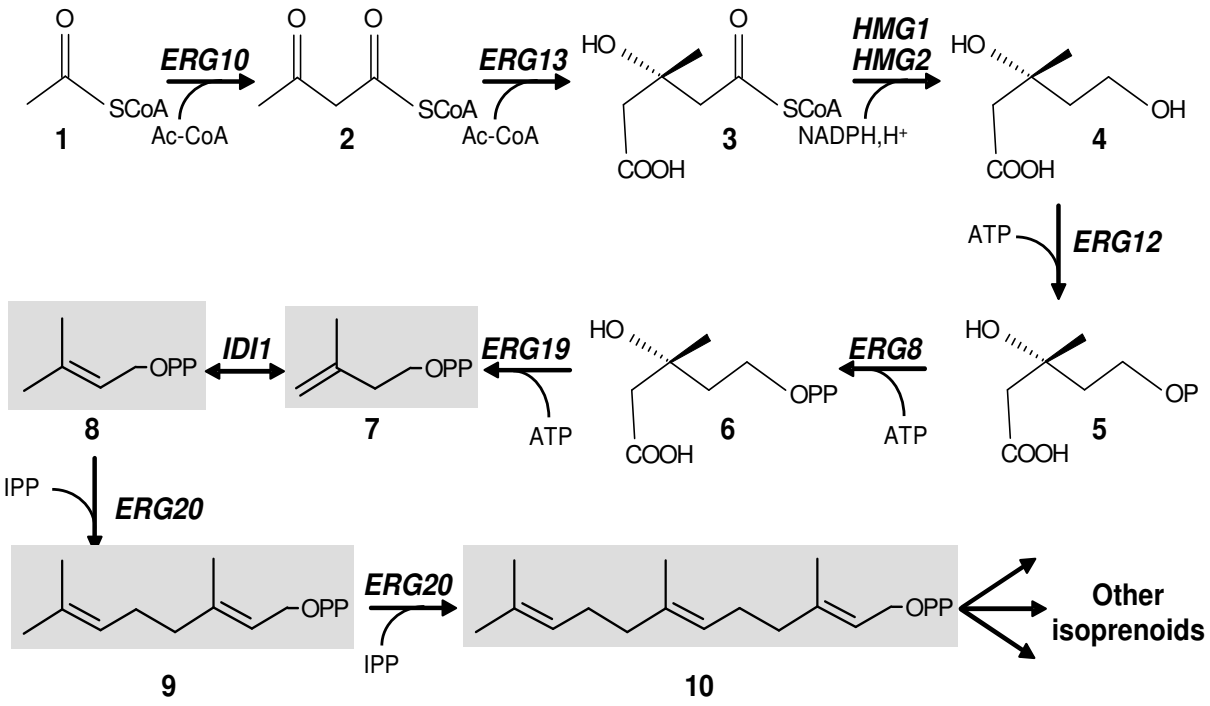

Figure 1.2. The mevalonate pathway of *S. cerevisiae*

1: acetyl-CoA, **2**: acetoacetyl-CoA, **3**: 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), **4**: mevalonate, **5**: phosphomevalonate, **6**: diphosphomevalonate, **7**: IPP, **8**: DMAPP, **9**: GPP, **10**: FPP. Gray boxes specify the general precursors for the different classes of isoprenoids.
ERG10: acetoacetyl-CoA thiolase, *ERG13*: HMG-CoA synthase, *HMG1*, *HMG2*: HMG-CoA reductases, *ERG12*: mevalonate kinase, *ERG8*: phosphomevalonate kinase, *ERG19*: diphosphomevalonate decarboxylase, *IDI1*: IPP:DMAPP isomerase, *ERG20*: FPP synthase.

The first reaction of the mevalonate pathway is the synthesis of acetoacetyl-CoA from two molecules of acetyl-CoA, catalyzed by the acetoacetyl-CoA thiolase which is encoded by *ERG10* (Figure 1.2). *S. cerevisiae* contains two forms of the enzyme, which have different subcellular locations (the cytosol and the mitochondrion). In *S. cerevisiae*, the reaction step is subject to regulation by the intracellular levels of sterols, by transcriptional regulation mediated by late intermediate(s) or product(s) of the pathway (Trocha and Sprinson, 1976; Servouse and Karst, 1986; Dimster-Denk and Rine, 1996; Dixon et al., 1997; Dimster-Denk et al., 1999). However, overexpression of *ERG10* did not increase the radio-labeled acetate incorporation on total sterol, suggesting other enzyme(s) of the sterol biosynthetic pathway to be flux controlling (Dimster-Denk and Rine, 1996).

The condensation of acetyl-CoA with acetoacetyl-CoA to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is catalyzed by the *ERG13* gene product, HMG-CoA synthase. This enzymatic step is subject to regulatory control (Trocha and Sprinson, 1976; Servouse and Karst, 1986). The details of the regulatory mechanism involved remain uncharacterized (Daum et al., 1998). However, the first crystal structure of an HMG-CoA synthase from an organism, *Staphylococcus aureus*, was recently described (Campobasso et al., 2004). Although the staphylococcal and streptococcal enzymes exhibit little identity (20%) with the eukaryotic counterparts, the amino acid residues involved in the acetylation and condensation reactions are conserved among bacterial and eukaryotic HMG-CoA synthases (Campobasso et al., 2004).

The third enzyme in the pathway, HMG-CoA reductase, responsible for the conversion of HMG-CoA into mevalonate, is the most studied enzyme of the mevalonate pathway. Unlike humans, *S. cerevisiae* has two copies of the gene encoding HMG-CoA reductase: *HMG1* and *HMG2*, but Hmg1p was shown to be responsible for more than 83% of the enzyme activity in wild type cells (Basson et al., 1986). Disruption of both genes is lethal for cell, as predicted. This enzymatic step is highly regulated at different levels and appears to be a key regulatory point in the mevalonate pathway.

Mevalonate kinase, encoded by *ERG12*, phosphorylates mevalonate at the C-5 position using ATP. It has been shown that FPP and geranyl diphosphate (GPP) exert an inhibitory effect on the enzyme (Dorsey and Porter, 1968). The next step catalyzed by the phosphomevalonate kinase, the gene product of *ERG8*, is not subject to feedback regulation by ergosterol (Daum et al., 1998). Overexpression of *ERG8* using the strong *GAL1* promoter led to largely unchanged ergosterol levels, suggesting that this enzyme is not flux controlling for ergosterol production (Lees et al., 1999).

The next two steps in the mevalonate pathway involve the *ERG19* gene product (mevalonate diphosphate decarboxylase) which converts mevalonate diphosphate to IPP. The *IDI1* gene product (isopentenyl diphosphate:dimethylallyl diphosphate isomerase) can then convert IPP into dimethylallyl diphosphate (DMAPP). IPP isomerase catalyzes an essential activation step in isoprenoid metabolism in the conversion of IPP to DMAPP by enhancing the electrophilicity of the isoprene unit by at least a billion-fold (Anderson et al., 1989). Two different classes of IPP isomerases have been reported: the type I enzyme, first characterized in the late 1950s, is widely

distributed in eukaryota and eubacteria, while the type II enzyme was recently discovered in *Streptomyces* sp. strain CL190 and in the archaeon *Methanothermobacter thermautotrophicus* (Barkley et al., 2004; Kaneda et al., 2001). The type I and type II isomerases have different structures and different cofactor requirements, suggesting that they catalyze isomerizations by different chemical mechanisms (Barkley et al., 2004). The properties of mevalonate diphosphate decarboxylase and of IPP isomerase are largely uncharacterized. However, reduced sterol content observed after overexpression of *ERG19* was attributed to the accumulation of diphosphate intermediates leading to feedback inhibitions. Hence, *ERG19* could encode a flux controlling step of the mevalonate pathway (Bergès et al., 1997).

The final step in the early portion of the pathway is the conversion of DMAPP into geranyl and farnesyl diphosphates (GPP and FPP, respectively). Farnesyl (geranyl) diphosphate synthase, the product of the *ERG20* gene, catalyzes this reaction. The enzyme combines first DMAPP and IPP to form GPP, and then GPP is extended by combination with a second IPP to form FPP. FPP synthase is a well characterized prenyltransferase. The enzyme has been purified to homogeneity from several eukaryotic sources including *S. cerevisiae* (Eberhardt and Rilling, 1975), avian liver (Reed and Rilling, 1975), porcine liver (Bernard et al., 1978; Yeh and Rilling, 1977) or human liver (Bernard and Popjak, 1981). FPP is a pivotal molecule situated at the branch point of several important metabolic pathways leading to sterols, heme, dolichol or quinone biosynthesis and prenylation of proteins and also involved in several key regulations of the mevalonate pathway. Furthermore, overexpression of *ERG20* has been shown to result in increased levels of enzyme activity and ergosterol production, indicating that FPP synthase exerts some flux control in the pathway (Daum et al., 1998).

The regulation of the isoprenoid biosynthetic pathway is known to be complex in all eukaryotic organisms examined, including the budding yeast *S. cerevisiae* (Goldstein and Brown, 1990; Hampton et al., 1996; Hampton, 1998). The overriding principle for the regulation of this pathway is multiple levels of feedback inhibition (Figure 1.3). This feedback regulation involves several intermediates and appears to act both at different steps of the pathway and at different levels of regulation as it involves changes in gene transcription, mRNA translation, enzyme activity and protein stability. The emerging picture is that the isoprenoid pathway has a number of points of regulation that act to control the overall flux through the pathway as well as the relative flux through the various branches of the pathway (Dimster-Denk et al., 1999). From

these complex multilevel regulations, two distinct but interconnected major sites of regulation are evident: one is the HMG-CoA reductase, the other is due to enzymes competing for FPP.

The yeast HMG-CoA reductase is subject to complex regulation by a number of factors and conditions, at different levels. At the transcriptional level, *HMG1* expression is stimulated by heme via the transcriptional regulator Hap1p while *HMG2* expression is inhibited, indicating a relationship between heme and sterol biosynthesis (Thorsness et al., 1989). Hmg1p was shown to be transcriptionally repressed by a non-sterol product of the pathway (Dimster-Denk et al., 1994). In a different study, the same group reported the induction of *HMG1* reporter gene after inhibition of squalene synthase or lanosterol demethylase suggesting that *HMG1* responded to the levels of sterol products of the pathway (Dimster-Denk et al., 1999). The two yeast isozymes have also distinctly different posttranslational fates: Hmglp was shown to be extremely stable while Hmg2p was subject to rapid regulated degradation depending on the flux through the mevalonate pathway (Hampton and Rine, 1994). The stability of each isozyme is determined by its non-catalytic amino-terminal domain. Hmg2p was demonstrated to undergo ERAD (endoplasmic reticulum associated degradation), similar to its mammalian ortholog, dependent on ubiquitination (Hampton and Rine, 1994; Nakanishi et al., 1988; Shearer and Hampton, 2004; Hampton and Bhakta, 1997). FPP was demonstrated as the source of the regulatory signal controlling and coupling ubiquitination/degradation of Hmg2p with the flux in the mevalonate pathway (Hampton and Rine, 1994; Hampton and Bhakta, 1997; Gardner and Hampton, 1999). In addition to the FPP signal, an oxysterol derived signal positively regulates Hmg2p degradation in yeast, but in contrast with mammals it is not an absolute requirement for degradation in yeast (Gardner et al., 2001).

To summarize, the different regulations of HMG-CoA reductase can be grouped as 1) feedback inhibition, i.e. regulation of HMG-CoA reductase activity in response to intermediates or products from the mevalonate pathway, and 2) cross-regulation, i.e. regulation by processes independent of the mevalonate pathway (Hampton et al., 1996). As a consequence, in aerobic conditions Hmg1p is actively synthesized and extremely stable consistent with the constant need of sterols, while in anaerobic conditions the enzyme with a high turn-over, i.e. Hmg2p, is dominant to allow rapid adjustment of the balance between cellular demand and the potential accumulation of toxic compounds (Hampton et al., 1996). *HMG1* and *HMG2* are also expressed differently as a function of the growth phase (Thorsness et al., 1989; Casey et al., 1992).

FPP, the product of FPP synthase (Erg20p), is a pivotal intermediate in the mevalonate pathway leading to the synthesis of several critical end products (Daum et al., 1998). In addition, the farnesyl units and the related geranyl and geranylgeranyl species are important elements for the posttranslational modification of proteins that require hydrophobic membrane anchors for proper placement and function. Furthermore, farnesol, a metabolite causing apoptotic cell death in human acute leukaemia, a molecule involved in quorum sensing in *Candida albicans* (Hornby et al., 2001; Grabińska and Palamarczyk, 2002) and causing growth inhibition in *S. cerevisiae*, is endogenously generated in the cells by enzymatic dephosphorylation of FPP (Haug et al., 1994; Melnykovych et al., 1992; Machida et al., 1998). To ensure a constant production of the multiple isoprenoid compounds at all stages of growth whilst preventing accumulation of potentially toxic intermediates, cells must precisely regulate the level of activity of enzymes of the mevalonate pathway (Brown and Goldstein, 1980). A number of experimental data show that biosynthesis of dolichols and ubiquinones, as well as isoprenylated proteins, is being regulated by enzymes distal to HMG-CoA reductase (Szkopińska et al., 2000; Grabowska et al., 1998). This is illustrated on one hand by recent data on the effects of modulating FPP pools on dolichol biosynthesis and on the other hand by effects of increased tRNA prenylation on FPP synthase levels.

In aerobic conditions, a strain with *ERG20* on a multicopy plasmid was characterized by an almost 6-fold higher FPP synthase activity than a control wild-type strain. Simultaneously, the HMG-CoA reductase activity was changed by about 20%, which is consistent with the known regulations of HMG-CoA reductase activity (Szkopińska et al., 2000). Such an immense increase in FPP synthase activity correlated with a significant elevation in dolichol and ergosterol synthesis (about 80% and 32% higher, respectively). These results suggested that FPP synthase, independently of HMG-CoA reductase, is responsible for the partition of FPP, the substrate for squalene synthase and cis-prenyltransferase, between the syntheses of both groups of compounds acting as a flux controlling enzyme (Szkopińska et al., 2000). An intricate correlation between FPP synthase activity, ergosterol level and physiology of the cells has also been observed (Karst et al., 2004). Nevertheless, the disruption of the squalene synthase gene (when the strain deleted of *ERG9* was cultivated in the presence of ergosterol) resulted in concurrently diminished activities of both FPP synthase and HMG-CoA reductase (78 and 83% repression, respectively). This strongly indicated the implication of squalene synthase in determining the intermediate flow

rates in the mevalonate pathway i.e. when the early intermediates of the pathway cannot be converted to ergosterol and its esters, and synthesis of dolichols is unable to assimilate the bulk of FPP, both FPP synthase and HMG-CoA reductase are repressed (Szkopińska et al., 2000). Moreover, changing an *erg9* deleted strain from a medium containing ergosterol to a medium deprived of ergosterol resulted in a more than 10-fold increase in FPP synthase activity while HMG-CoA reductase activity was increased by 1.4-fold. Therefore, evidence is given that earlier literature data indicating strictly coordinated regulation of the mevalonate pathway enzymes, i.e. HMG-CoA reductase, FPP synthase, and squalene synthase with HMG-CoA reductase as the main regulatory enzyme in sterol biosynthesis, does not find full confirmation. FPP synthase, independently of HMG-CoA reductase and to a certain degree of squalene synthase, responds the most to changes in internal and external environmental conditions (Szkopińska et al., 2000). This is perhaps not surprising if one considers the diversified cell functions in which its product, FPP, directly participates (Szkopińska et al., 2000).

DMAPP, the substrate of FPP synthase, forms a branch point of the isoprenoid pathway because it is also a substrate of Mod5p, tRNA isopentenyltransferase (Gillman et al., 1991). As a consequence, tRNA and the isoprenoid biosynthetic pathway compete for DMAPP as a common substrate. It has been shown that overexpression of *ERG20* causes a decrease of i^6A modification of tRNA, i.e. tRNA processing is dependent upon changes in the level of FPP synthase (Kamińska et al., 2002). Moreover, in a strain defective in Maf1p (a negative regulator of tRNA transcription) an excessive amount of DMAPP is dedicated to tRNA modification and, consequently, a lower amount of DMAPP is accessible for FPP synthase. As a consequence, the *maf1-1* strain is characterized by elevated levels of Erg20p and decreased ergosterol content. In this case, regulation of Erg20p levels is both due to transcriptional as well as post-translational regulations (Kamińska et al., 2002). Therefore, in yeast, tRNA levels appear to contribute to the complex regulation of FPP synthase and of the mevalonate pathway.

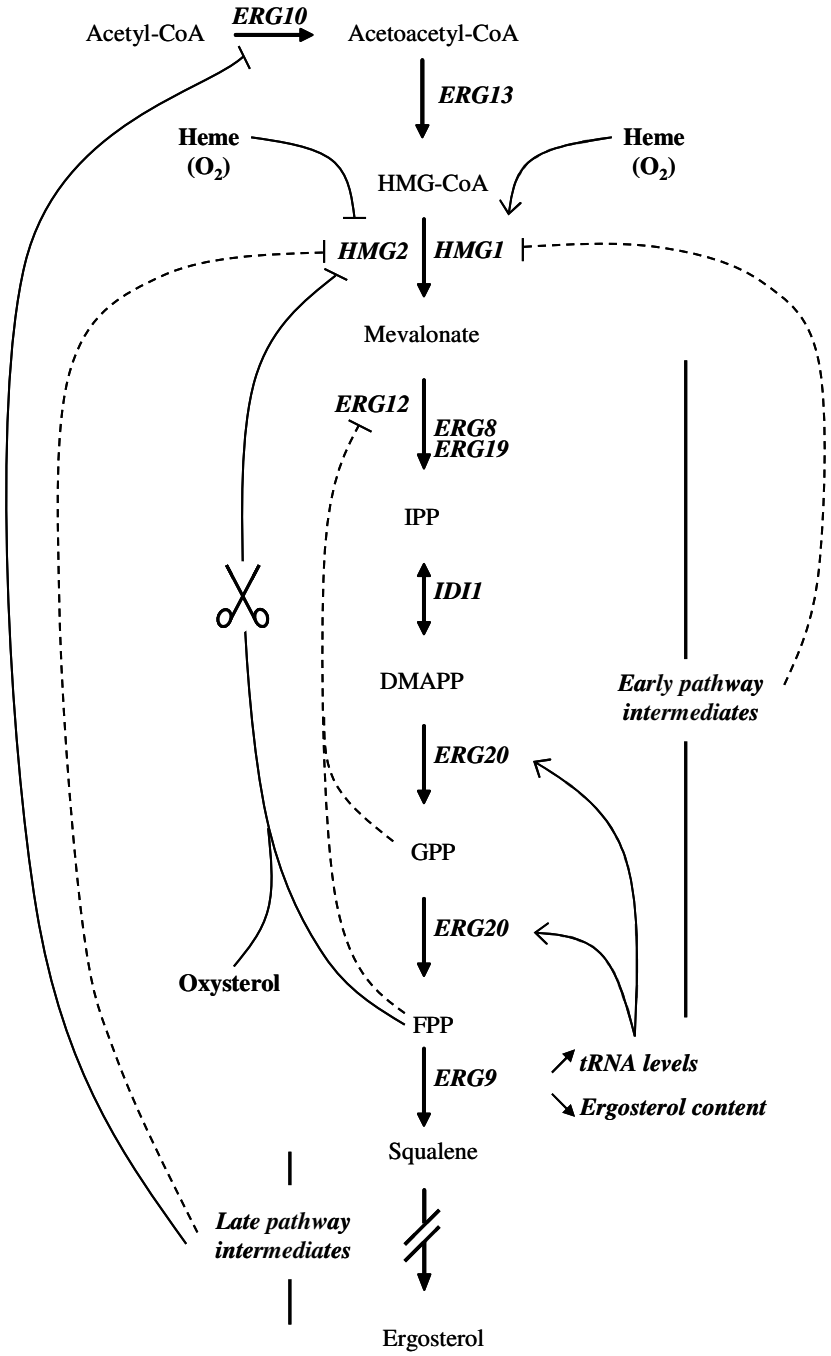

Figure 1.3. Principal regulations of the mevalonate pathway
Solid lines: regulations at gene expression level, dashed lines: regulations at protein synthesis level, ✂: regulation of protein stability.

1.3 The MEP pathway

Since the discovery of the mevalonate pathway, it was largely accepted that IPP and DMAPP originated exclusively from this pathway in all living organisms. However, inconsistency of several results, mainly involving labeling experiments, with the sole operation of the mevalonate pathway was reported (Zhou and White, 1991; Cane et al., 1979; Cane et al., 1981; Flesch and Rohmer, 1988). The existence of a second pathway was discovered relatively recently by the research groups of Rohmer and Arigoni using stable isotope incorporation in various eubacteria and plants (Rohmer et al., 1993; Schwarz, 1994). These data suggested that pyruvate and a triose phosphate could serve as precursors for the formation of IPP and DMAPP (Rohmer et al., 1993). The gene encoding the first reaction step of the alternative non-mevalonate pathway was identified and cloned from *E. coli* and the plant *Mentha piperita* (Sprenger et al., 1997; Lois et al., 1998; Lange et al., 1998) (Figure 1.4). It now seems obvious that most Gram negative bacteria and *Bacillus subtilis* use the MEP pathway for isoprenoid biosynthesis, whereas staphylococci, streptococci, enterococci, fungi and archae use the mevalonate pathway (Wilding et al., 2000; Hedl et al., 2002; Bochar et al., 1999; Doolittle and Logsdon, 1998). Although most *Streptomyces* strains are equipped with the MEP pathway, some of them have been reported to possess the mevalonate pathway in addition to the MEP pathway to produce terpenoid antibiotics (Takagi et al., 2000a; Hamano et al., 2001; Hamano et al., 2002; Kawasaki et al., 2003). *Listeria monocytogenes* was reported as the only pathogenic bacterium known to contain both pathways concurrently (Begley et al., 2004). Plants use the MEP pathway in plastids and the mevalonate pathway in their cytosol. Elucidation of the MEP pathway has been achieved through multidisciplinary approaches including organic chemistry, microbial genetics, biochemistry, molecular biology, and bioinformatics. The impressive fast increase in available information about the MEP pathway is a good example of integration of genomics with more traditional approaches to identify whole metabolic pathways in distant organisms (Rodríguez-Concepción and Boronat, 2002).

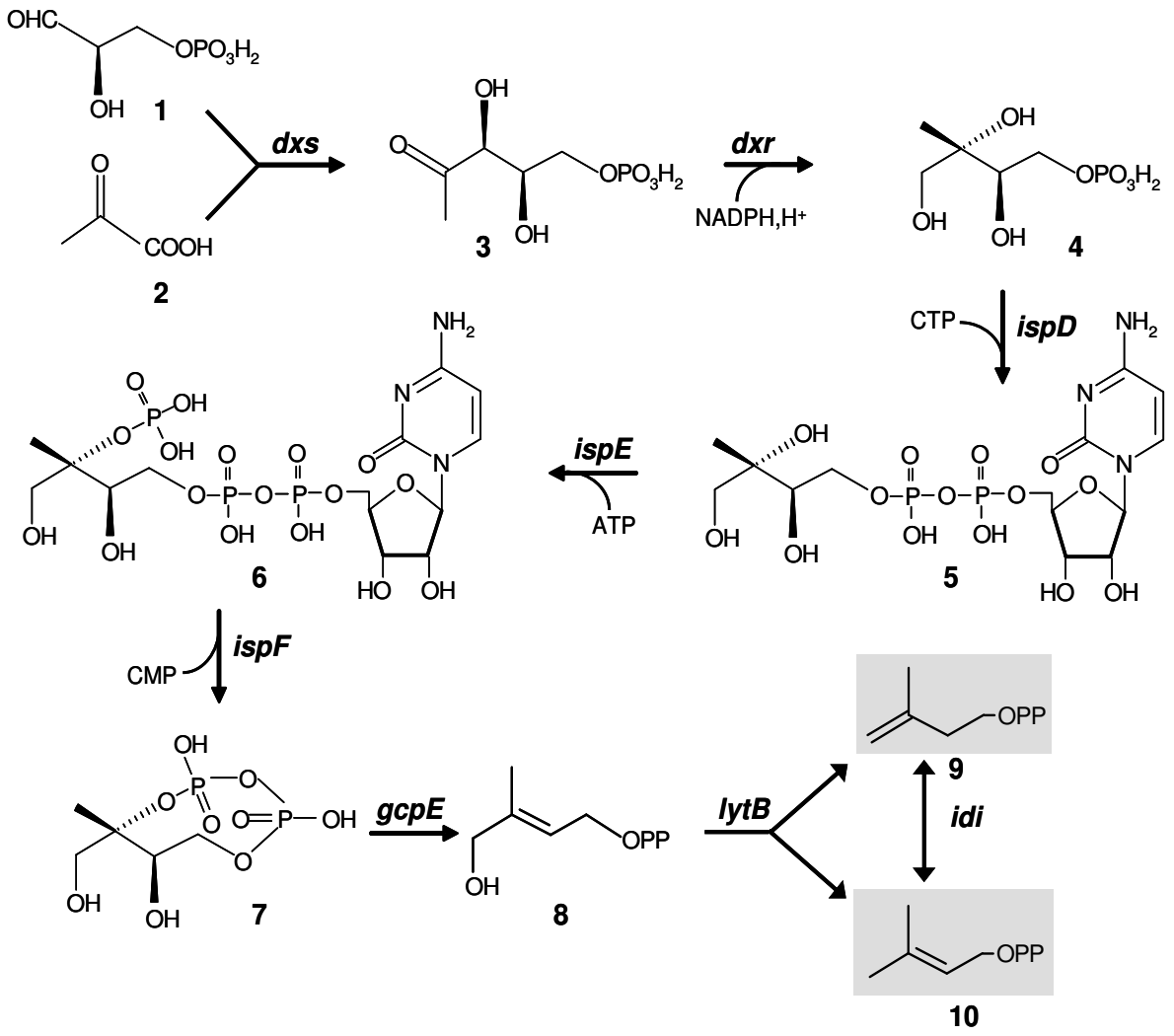

Figure 1.4. The *E. coli* MEP pathway for the synthesis of IPP and DMAPP.

1: D-glyceraldehyde 3-phosphate, **2**: pyruvate, **3**: 1-deoxy-D-xylulose 5-phosphate, **4**: 2-C-methyl-D-erythritol 4-phosphate, **5**: 4-diphosphocytidyl-2-C-methyl-D-erythritol, **6**: 2-phospho-4-diphosphocytidyl-2-C-methyl-D-erythritol, **7**: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, **8**: 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate, **9**: isopentenyl diphosphate, **10**: dimethylallyl diphosphate.

The enzymes encoded by the different genes are: *dxs*: DXP synthase, *dxr*: DXP isomeroreductase, *ispD*: MEP cytidylyltransferase, *ispE*: CDP-ME kinase, *ispF*: MECDP synthase, *gcpE*: MECDP reductase, *lytB*: HMBPP reductase.

In the first step of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate synthase, also named DXP synthase or Dxs, catalyzes the condensation of the two precursors from the central metabolism, D-glyceraldehyde 3-phosphate (GAP) and pyruvate, to form DXP. However, DXP synthase is not the first specific enzymatic step of the MEP pathway as, in addition to IPP and DMAPP, DXP is the precursor for the biosynthesis of vitamins B1 (thiamine) and B6 (pyridoxal) in *E. coli* (Sprenger et al., 1997). DXP synthase activity, which is relatively high compared to the other enzymes of the pathway, requires both thiamine and a divalent cation (Mg^{2+} or Mn^{2+}) (Eisenreich et al., 2004). DXP synthases represent a new class of TPP dependent enzymes combining the characteristics of decarboxylases and transketolases (Eubanks and Poulter, 2003).

As DXP is the precursor for different kinds of compounds, the committed step of the pathway is catalyzed by DXP isomeroreductase (Dxr) and leads to the formation of 2-C-methyl-D-erythritol 4-phosphate (MEP), hence its name: "MEP pathway". *yaeM* gene from *E. coli* was shown to be responsible for the rearrangement and reduction of DXP in a single step (Takahashi et al., 1998) and was therefore renamed *dxr*. The catalytic activity of DXP isomeroreductase is substantially lower (12 μmol mg^{-1} min^{-1}) than DXP synthase (Eisenreich et al., 2004). The reaction catalyzed by DXP isomeroreductase is reversible although the equilibrium is largely displaced in favor of the formation of MEP (Hoeffler et al., 2002a). Due to the wide distribution of DXP isomeroreductase in plants and many eubacteria, including pathogenic bacteria, and its absence in mammalian cells, this enzyme has been studied as a target for herbicides and antibacterial drugs. Fosmidomycin, an antibacterial agent active against most Gram negative and some Gram positive bacteria, has been shown to be a strong specific and competitive inhibitor of DXP isomeroreductase activity (Takahashi et al., 1998).

In order to study the MEP pathway, *E. coli* strains were engineered to allow the study of mutations in otherwise essential genes. For this purpose, in addition to the MEP pathway, *E. coli* was transformed with the genes encoding yeast mevalonate kinase, phosphomevalonate kinase and diphosphomevalonate decarboxylase in one operon. This allowed the study of mutants of the MEP pathway which would have led to lethality of wild type cells (Kuzuyama, 2002; Campos et al., 2001a). Mutants with a defect in the synthesis of IPP from MEP were isolated and the genes responsible for this defect identified. These genes are *ygbP*, *ychB*, *ygbB* and *gcpE*. The genes *ygbP*, *ychB*, and *ygbB* are all essential in *E. coli* and the enzymatic steps catalyzed by their gene products belong to the trunk line of the MEP pathway (Campos et al., 2001a).

ygbP (*ispD*) was shown to encode MEP cytidylyltransferase converting MEP into 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) in the presence of CTP (Rohdich et al., 1999; Kuzuyama et al., 2000a). Its activity is also substantially lower compared to DXP synthase activity.

In the presence of ATP, CDP-ME is converted to 2-phospho-4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME2P) by the CDP-ME kinase encoded by *ispE* (Lüttgen et al., 2000; Kuzuyama et al., 2000b). On the basis of sequence comparisons, CDP-ME kinase was recognized as a member of the GHMP kinase family which initially included galactose kinase, homoserine kinase, mevalonate kinase and phosphomevalonate kinase and more recently mevalonate 5-diphosphate decarboxylase and the archaeal shikimate kinase (Miallau et al., 2003).

2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP) synthase, encoded by *ygbB* (*ispF*), was demonstrated to catalyze the formation of MECDP from CDP-ME2P with concomitant elimination of cytidine monophosphate (CMP) (Takagi et al., 2000b; Herz et al., 2000). *ispF* has been shown to be essential (Campos et al., 2001a; Freiberg et al., 2001) and conditional mutation of *ispF* in *E. coli* or of its ortholog *yacN* in *B. subtilis* led to a decrease in growth rate and altered cell morphology (Campbell and Brown, 2002). In contrast to the dispersed nature of genes belonging to the MEP pathway, *ispD* and *ispF* are transcriptionally coupled or, in some cases, fused into one coding region leading to a bifunctional enzyme. IspDF coupling is highly unusual as these enzymes catalyze non consecutive steps of the MEP pathway. Interactions have been observed between the bifunctional IspDF and IspE protein. Monofunctional IspD, IspF and IspE proteins have also demonstrated a close interaction suggesting a multienzymatic complex maybe responsible for the metabolic flux control through the MEP pathway (Gabrielsen et al., 2004).

In contrast to the mevalonate pathway, in which DMAPP is synthesized from IPP by the essential IPP:DMAPP isomerase activity, the finding that IPP:DMAPP isomerase was functional but non-essential for growth of *E. coli* indicated that the MEP pathway was branched i.e. DMAPP and IPP are synthesized by two different routes splitting at late stages of the pathway (Rodríguez-Concepción et al., 2000).

The last two steps of the pathway were recently solved by Hintz et al. (2001) who reported the accumulation of the formerly unknown intermediate 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP) in a *lytB* (*ispH*) disrupted *E. coli* strain. Several studies aimed at

demonstrating the essential nature of *gcpE* (*ispG*) and/or *lytB* (Altincicek et al., 2001a; Altincicek et al., 2001b), their necessity for DXP conversion to IPP and DMAPP (Hecht et al., 2001; Steinbacher et al., 2002; Campos et al., 2001b) and the efficiency of their gene products in converting MECDP into HMBPP (Seemann et al., 2002) and HMBPP into IPP and DMAPP (Altincicek et al., 2002). An important feature of both GcpE and LytB is a [4Fe-4S] cluster as prosthetic group underlying their high sensitivity towards oxygen. This property, common to both enzymes, may explain why the investigations of the terminal reactions of the MEP pathway have been hampered for so long (Seemann et al., 2002; Eberl et al., 2003; Wolff et al., 2003).

The finding that a single enzyme is responsible for the formation of both IPP and DMAPP contrasts with the mevalonate pathway where DMAPP is successively formed from IPP by IPP isomerase. As a consequence of these findings, the role of IPP isomerase in microorganisms expressing the MEP pathway comes into question. The non-essential and non-limiting roles of the Idi interconversion are currently being investigated as on one hand the *E. coli* Idi enzyme was reported to have 20-fold less activity than its yeast counterpart (Hahn et al., 1999), *idi* from *E. coli* is dispensable (Rodríguez-Concepción et al., 2000) and *idi* homologs have not been found in genomes of many bacteria using the MEP pathway sequenced so far (Cunningham et al., 2000). On the other hand, structurally and mechanistically different IPP isomerases, referred to as class II IPP isomerases, have been identified in *Streptomyces* sp. strain CL190 and also in a variety of Gram-positive bacteria, cyanobacteria and archaebacteria (Hamano et al., 2001). Furthermore, the overexpression of *idi* genes from different origins in *E. coli* engineered for the production of lycopene has always led to carotenoid overproduction (Cunningham et al., 2000; Kajiwara et al., 1997; Wang et al., 1999), these findings keep going the debate about the non-essentiality and non-limiting role of IDI reaction (Hoeffler et al., 2002b).

1.4 Metabolic engineering of microorganisms for isoprenoid production

In the last decade there have been a number of investigations on the construction of engineered microorganisms with the ability to produce different isoprenoids. Figure 1.5 schematically shows the different steps for constructing industrial isoprenoid producing microorganisms. As it will be seen in the next sections, a common feature for most of the conducted studies for microbial isoprenoid production is that they include expression of heterologous genes for converting

isoprenoid precursors of the host microorganism into the desired isoprenoid, and deregulation of metabolic pathways in order to increase the metabolic flux to isoprenoid precursors.

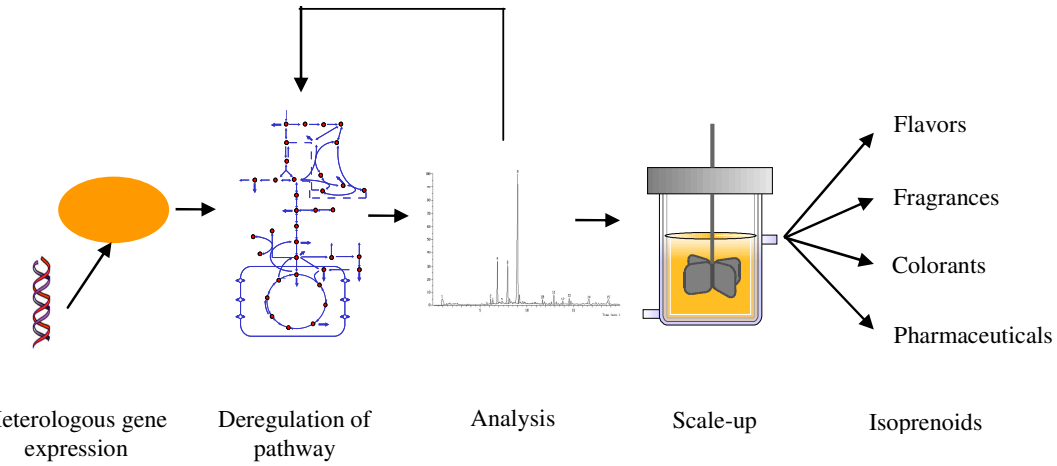

Figure 1.5. Summary of different steps for establishing industrial cell factories capable of isoprenoid production

Tetraterpenoid carotenoids (C_{40}) have been the most interesting group of isoprenoids for metabolic engineering studies because of their easy color screening (Marshall and Wilmoth, 1981) and their industrial importance as feed supplements in the poultry and fish farming (Johnson and Schroeder, 1996). Carotenoid biosynthetic pathway in *Erwinia uredovora* was first elucidated by Misawa et al (1990), and the corresponding genes were subsequently used in several studies for production of heterologous carotenoids in non-carotenogenic microorganisms. However, isolation and characterization of more than 150 carotenogenic genes involved in the synthesis of 27 different enzymes in the carotenoid biosynthesis pathways in several organisms (Lee and Schmidt-Dannert, 2002; Schmidt-Dannert, 2000) has opened the door for the heterologous production of a broad range of carotenoids.

Ergosterol (the main sterol in yeasts), found in large amounts in the yeast membranes, plays a key role in regulating the membrane fluidity and permeability (Arthington-Skaggs et al., 1996) and is produced through the mevalonate pathway. Although *E. coli* has been the main host for metabolic engineering of isoprenoids, in several cases yeasts (which have high capacity for ergosterol production) have been subject to metabolic engineering studies (Yamano et al., 1994; Miura et al., 1998a; Miura et al., 1998b; Shimada et al., 1998; Jackson et al., 2003; Verwaal et

al., 2007, Takahashi et al., 2007; Lindahl et al., 2006; Ro et al., 2006; Shiba et al., 2007; DeJong et al., 2005; Oswald et al., 2007).

1.4.1 Metabolic engineering of the MEP pathway

Amongst the different enzymes in the MEP pathway, DXP synthase (encoded by *dxs*), IPP isomerase (encoded by *idi*) and DXP isomeroreductase (encoded by *dxr*) have been the main targets for the metabolic engineering investigations. Overexpression of *dxs* has been achieved in several studies in order to improve the intracellular pool of precursors for isoprenoid biosynthesis (Huang et al., 2001; Reiling et al., 2004; Martin et al., 2001; Martin et al., 2003; Wang and Liao, 2000; Matthews and Wurtzel, 2000; Harker and Bramley, 1999; Kim and Keasling, 2001; Albrecht et al., 1999; Yuan et al., 2006; Yoon et al., 2007; Alper et al., 2005c). For example, overexpression of *dxs* in *E. coli* strains harboring the carotenogenic genes resulted in up to 10.8- and 3.9-fold increase in the accumulated levels of lycopene and zeaxanthin, respectively (Matthews and Wurtzel, 2000). Overproduction of DXP synthase also had a great impact on the biosynthesis of taxadiene (Huang et al., 2001) as the required intermediate for the synthesis of paclitaxel (Taxol), and lycopene in *E. coli* (Harker and Bramley, 1999). Chromosomal replacement of native *dxs* promoter with a strong bacteriophage T5 promoter improved β-carotene production 2- to 3.3-fold in *E. coli* (Yuan et al., 2006). The strength of promoter and plasmid copy number are important in balancing expression of *dxs* with overall metabolism (Kim and Keasling, 2001).

The second step in the MEP pathway, which is catalyzed by DXP isomeroreductase, has been shown to control the flux to isoprenoid precursors in *E. coli* (Kim and Keasling, 2001; Albrecht et al., 1999). Co-overexpression of *dxr* and *dxs* was concomitant with a 1.4- to 2-fold increase in lycopene level compared to the strains overexpressing only *dxs* (Kim and Keasling, 2001). However, overexpression of *dxs* had a greater impact on lycopene production than overexpression of *dxr*. In another study (Albrecht et al., 1999), simultaneous overexpression of *dxs* and *dxr* in the ß-carotene and zeaxanthin producing *E. coli* strains was lethal for the cells, probably due to restricted storage capacity for lipophilic carotenoids, which causes membrane overload and loss of its functionality. This problem implies the need for host microorganisms with higher storage capacity for heterologous production of carotenoids (Sandmann et al., 1999; Sandmann, 2001; Ruther et al., 1997). However, in contrast to the previous study (Albrecht et al.,

1999) that showed 1.6 mg/g DW as the maximum membrane capacity of *E. coli* for ß-carotene and zeaxanthin storage, Yoon et al. (2006) did not observe any toxicity effect in the *E. coli* strains accumulating 49.3 mg ß-carotene/g DW.

Isomerization of IPP to DMAPP has been another target for improving isoprenoid biosynthesis in the MEP pathway, and several studies have shown the enhancing effect of IPP isomerase overproduction (Kajiwara et al., 1997; Wang et al., 1999; Huang et al., 2001; Reiling et al., 2004; Martin et al., 2003; Albrecht et al., 1999; Yuan et al., 2006). Overexpression of *idi* genes from different organisms in the recombinant *E. coli* showed 1.5- to 4.5-fold increase in the lycopene, ß-carotene, and phytoene levels compared to the control strains (Kajiwara et al., 1997). Positive effects of *idi* or *dxs* overexpression on ß-carotene and zeaxanthin accumulation in *E. coli* have also been shown. Amplification of *idi* or/and *dxs* resulted in approximately 2- to 3.5-fold more carotenoid accumulation in the recombinant strains than the control (Albrecht et al., 1999; Yuan et al., 2006). Engineered lycopene producing *E. coli* overexpressing *dxs, idi,* and *ispA* (responsible for FPP synthase activity in *E. coli*) produced 6-fold more lycopene than the control strain (Reiling et al., 2004). Simultaneous amplification of *idi* and GGPP synthase gene (*gps*) in astaxanthin producing *E. coli* strains increased the astaxanthin level from 33 μg/g dry weight in the control strain to 1419 μg/g dry weight in the recombinant strain (Wang et al., 1999). In the same laboratory, subjecting *gps* gene to directed evolution resulted in 2-fold increase in the lycopene level and subsequent co-overexpressions of *dxs* gene further enhanced the lycopene accumulation (Wang et al., 2000).

The MEP pathway is initiated with combining pyruvate and GAP in equal amounts catalyzed by DXP synthase. Hence, balanced pools of pyruvate and GAP would be an important factor in efficient directing of central carbon metabolism to the isoprenoid pathway. Pyruvate is required as a precursor in many cellular pathways and presumably it is more available than GAP for isoprenoid biosynthesis. It was shown that overproduction or inactivation of enzymes that leads to redirection of flux from pyruvate to GAP results in higher lycopene production in *E. coli* (Farmer and Liao, 2001). Thus, overproduction of phosphoenolpyruvate (PEP) synthase (Pps) and PEP carboxykinase (Pck) or inactivation of pyruvate kinase isozymes (Pyk-I and Pyk-II) were shown to enhance lycopene production in *E. coli*.

Effect of manipulating central carbon metabolism on lycopene production in *E. coli* by inactivating the competing pathways at acetyl-CoA and pyruvate nodes has been studied (Vadali,

et al., 2005). Deletion of acetate production pathway at pyruvate node improved lycopene production by 45% but further deletion of acetate production pathway at acetyl-CoA node and lactate production pathway did not have any more effect.

Poor expression of plant genes and inadequate amounts of enzymes could be another limiting factor for the production of plant isoprenoids in the engineered hosts (Martin et al., 2001). To circumvent the problems of low sesquiterpene yield arisen from the poor expression of plant genes, in one study (Martin et al., 2003), a codon-optimized variant of amorphadiene synthase gene (*ADS*) was synthesized and expressed in *E. coli*. This improved the enzyme synthesis and production yield of amorphadiene and changed the flux control in the biosynthesis of sesquiterpenes from the step catalyzed by the heterologous plant genes to the supply of precursor (FPP) provided by the MEP pathway. The expression of this synthetic *ADS* gene in *E. coli* resulted in a 10- to 300-fold increase in sesquiterpene accumulation compared to the previous study (Martin et al., 2001) in which the native plant sesquiterpene synthase genes were expressed. Further overexpression of genes responsible for the synthesis of DXP synthase, IPP isomerase and FPP synthase, with the synthetic *ADS* led to a 3.6-fold increase in the concentration of amorphadiene indicating the supply of precursor is limiting the sesquiterpene production. However, considering the fact that overexpression of three flux controlling enzymes of the pathway only resulted in a 3.6-fold increase in amorphadiene concentration, this approach to increase the flux to FPP seems to be limited by some other native control mechanisms in *E. coli*. Introduction of the mevalonate pathway from *S. cerevisiae* to *E. coli* has been shown to be an alternative approach to increase the intracellular concentration of isoprenoid precursors, thereby circumventing the as yet unidentified regulations of the native MEP pathway and also minimizing the complicated regulatory network of the mevalonate pathway observed in yeast. The mevalonate pathway from *S. cerevisiae* was divided into two synthetic operons, referred to as top (MevT) and bottom (MBIS) section of the pathway and was expressed in the *E. coli* strain harboring the engineered MEP pathway and synthetic *ADS*. MevT contains three genes for the conversion of acetyl-CoA to mevalonate, whereas MBIS is responsible for the conversion of mevalonate to FPP. Importing the heterologous mevalonate pathway into *E. coli* resulted in 10-fold further increase in the amorphadiene concentration (Martin et al., 2003). This engineered strain produced 480 mg/L amorphadiene in a fed-batch bioreactor supplemented with increased carbon and complex nutrients (Newman et al., 2006). Growing the above strain in a medium

supplemented with increasing concentration of exogenous mevalonate led to higher amorphadiene production which indicates limited supply of mevalonate by the MevT operon. However, increasing expression of the MevT genes inhibited cell growth due to accumulation of HMG-CoA. Introduction of an additional copy of *tHMG1* for conversion of the toxic HMG-CoA to non-toxic mevalonate, restored the growth of cells, reduced the accumulation of intracellular HMG-CoA and improved mevalonate production 2-fold (Pitera et al., 2007). To remedy the imbalanced gene expression in the MevT genes, expression of the three genes in the operon was tuned by generating libraries of tunable intergenic regions (TIGRs) and resulted in a 7-fold increase in mevalonate production (Pfleger et al., 2006).

Heterologous expression of the mevalonate pathway from *Streptomyces* sp. Strain CL190 into lycopene producing *E. coli* improved lycopene biosynthesis only if acetate production pathway had been inactivated (Vadali et al., 2005). In another study (Yoon et al., 2006), the lower mevalonate pathway from the Gram-positive bacterium *Streptococcus pneumoniae* was imported into lycopene producing *E. coli*. Supplementation of 3.3 mM mevalonate for the mevalonate lower pathway improved lycopene production more than 3-fold. Cultivation of this strain in 2YT medium containing 2(w/v)% glycerol, 6.6 mM mevalonate and 0.5(w/v)% Tween 80 to prevent clump formation due to high amounts of lycopene resulted in 102 mg/L lycopene which is about 5-fold higher than the lycopene obtained through metabolic engineering of the MEP pathway by the same group (Kim and Keasling, 2001). The above engineered *E. coli* strain harboring the lower mevalonate pathway from *S. pneumoniae* was also studied for β-carotene production by introducing *crtY* gene from *Pantoea ananatis* and a 4-fold increase in β-carotene production was observed due to expression of the lower mevalonate pathway and mevalonate supplementation. Addition of 16.5 mM mevalonate and 2.5(w/v)% glycerol resulted in 503 mg/L (49.3 mg/g DW) β-carotene which is the highest reported microbial carotenoid production to date. In contrast to lycopene, β-carotene had no effect on cell growth and addition of Tween 80 was not necessary for preventing clump formation (Yoon et al., 2007).

Most of the metabolic engineering efforts on microbial isoprenoid production were focused on the genes that are directly involved in the isoprenoid biosynthesis pathway, genes from central carbon metabolism or introduction of heterologous mevalonate pathway. However, there have been some attempts to identify new target genes from other pathways that can affect isoprenoid biosynthesis. In one study (Alper et al., 2005a), genome-wide stoichiometric flux balance

analysis using a genome-scale model for *E. coli* and minimization of metabolic adjustment (MOMA) requirement between wild type and single gene knockout mutant as an objective function was performed to identify the genes whose deletions could enhance lycopene production in *E. coli*. Using this systematic (model-based) approach a triple knockout construct (*ΔgdhA ΔaceE ΔfdhF*) was identified that resulted in approximately 40% more lycopene production compared to the engineered parental strain. In a further improvement, the same group (Alper et al., 2005b) identified additional knockout targets by applying a global transposon library search (combinatorial method) in the parental strain. By combining the identified target genes from both systematic and combinatorial approaches, they found two globally optimum mutants each producing the same amounts of lycopene. One of the mutants (*ΔgdhA ΔaceE ΔfdhF*) was obtained using pure systematic approach and the other (*ΔgdhA ΔaceE* Δ_P*yjiD*) was a result of combination of targets from both approaches. Detailed characterization of these two mutants in a fed-batch fermenter running under optimized operating parameters revealed significant increase in the volumetric productivity and specific productivity for both engineered strains compared to the parental strain (Alper et al., 2006a).

Metabolic engineering of microbial hosts for constructing overproducers of a certain compound in many cases needs overexpression of genes to enhance the flux towards the product of interest. In one study (Kang et al., 2005), a colorimetric screening of shot-gun library clones with *E. coli* genomic library was used to identify target genes whose overexpressions could enhance lycopene production in *E. coli* lycopene producing strains. After analyzing 6 overproducer clones, 4 genes were identified to be involved in improved lycopene production. Among these 4 genes, 3 genes were not directly involved in lycopene biosynthesis and 2 of them were new target genes not identified by others.

Metabolic engineering of microorganisms for improving a certain phenotype often needs simultaneous alterations in the expression of many genes. This, in many cases is unachievable due to both difficulties in identifying all target genes using conventional pathway analysis methods and experimental limitations. Global transcription machinery engineering (gTME) approach (Alper et al., 2006b) as a means to induce multiple genetic perturbations through altering key proteins regulating the global transcriptome was applied to improve several phenotypes including lycopene production in *E. coli* (Alper and Stephanopoulos, 2007).

1.4.2 Metabolic engineering of the mevalonate pathway

Engineering of industrially important yeasts, *S. cerevisiae* and *Candida utilis*, for carotenoid production by introducing the carotenoid biosynthetic genes of *E. uredovora* has been reported (Yamano et al., 1994; Miura et al., 1998a; Miura et al., 1998b; Shimada et al., 1998; Verwaal et al., 2007). Modification of carotenogenic genes based on the codon usage of the *C. utilis* GAP dehydrogenase gene, increased the phytoene and lycopene contents of the strains 1.5- and 4-fold, respectively, compared to those of the strains carrying non-modified genes (Miura et al., 1998b). HMG-CoA reductase is believed to be the key enzyme in the mevalonate pathway and overexpression of both full length and truncated versions of the gene responsible for HMG-CoA reductase synthesis increased the lycopene production in *C. utilis* but the truncated version had greater impact. Subsequent disruption of *ERG9* gene also improved lycopene production (Shimada et al., 1998). Stimulating effect of HMG-CoA reductase overproduction on the lycopene and neurosporaxanthin content in a naturally carotenoid producing fungus, *Neurospora crassa* (Wang and Keasling, 2002), on β-carotene (Verwaal et al., 2007), epi-cedrol (Jackson et al., 2003) and amorphadiene (Ro et al., 2006) production in *S. cerevisiae* have also been shown.

Transformation of *S. cerevisiae* with carotenogenic genes from *E. uredovora* led to low levels of lycopene and β-carotene (Yamano et al., 1994), whereas *S. cerevisiae* transformed with the carotenogenic genes from *Xanthophyllomyces dendrorhous* (formerly known as *Phaffia rhodozyma*) produced higher levels of carotenoids (Verwaal et al., 2007). Overexpression of *tHMG1* enhanced carotenoid production 7-fold but phytoene was the main produced carotenoid suggesting existence of a bottleneck at the phytoene desaturation step. Overexpression of *crtI* changed the composition of carotenoids in the favor of β-carotene and led to reduced accumulation of phytoene (Verwaal et al., 2007).

Heterologous production of several sesquiterpenes including epi-cedrol (Jackson et al., 2003), 5-epi-aristolochene, premnaspirodiene, valencene, capsidiol (Takahashi et al., 2007), amorphadiene (Lindahl et al., 2006; Ro et al., 2006), and artemisinic acid (Ro et al., 2006) in *S. cerevisiae* have been successfully reported. In one study (Ro et al., 2006), *S. cerevisiae* was exploited as a platform for the biosynthesis of artemisinic acid, which can readily be converted to the antimalarial drug artemisinin by semisynthetic routes (Ro et al., 2006). To this end, first the mevalonate pathway in *S. cerevisiae* was deregulated by several modifications including overexpression of a truncated HMG-CoA reductase, down-regulation of *ERG9*, overexpression

of *upc2-1*, a semi-dominant allele that enhances the activity of a global transcription factor regulating sterol biosynthesis in yeast encoded by *UPC2*, and overexpression of *ERG20*. Combining all these alterations resulted in an engineered yeast strain capable of producing 153 mg/L amorphadiene. This engineered strain was subsequently transformed with a novel cytochrome P450 monooxygenase (*CYP71AV1*) from *Artemisia annua* that converts amorphadiene to artemisinic acid through a three-step oxidation and was able to produce up to 100 mg/L artemisinic acid (Ro et al., 2006). Engineering of pyruvate dehydrogenase bypass by overproduction of acetaldehyde dehydrogenase (encoded by *ALD6*) and a variant of acetyl-CoA synthetase from *Salmonella enterica* enhanced amorphadiene biosynthesis in *S. cerevisiae* (Shiba et al., 2007).

S. cerevisiae has been examined as a microbial host for the biosynthesis of Taxol intermediates as well (DeJong et al., 2005). Total biosynthesis of Taxol from GGPP takes place in 19 steps. Eight genes of the pathway were functionally expressed in the yeast. Co-expression of GGPP synthase and taxadiene synthase resulted in a yeast strain producing 1 mg/L taxadiene but further modification of this terpene backbone was limited by the first cytochrome P450 hydroxylation step and produced only 25 µg/L of taxadien-5α-ol.

GPP serves as the sole precursor for the biosynthesis of monoterpenes. The level of GPP in yeast seems to be very low due to its tight binding to the farnesyl diphosphate synthase catalytic site and also the fact that no cellular function for GPP has been described in yeast. However, expression of geraniol synthase of *Ocimum basilicum* in yeast gave rise to the biosynthesis of geraniol and linalool in yeast. A 10-fold increase in geraniol production was observed upon expression of geraniol synthase in a yeast mutant bearing an *erg20-2* mutation (Oswald et al., 2007).

Table 1.3 summarizes the examples of metabolically engineered microorganisms for production of different isoprenoids.

Table 1.3 Examples of different isoprenoids produced by metabolically engineered microorganisms

Class	Isoprenoid	Host microorganism	Yield/ concentration	Ref.
Monoterpenoids	Limonene	*E. coli*	~ 5000 µg/L	Carter et al., 2003
	3-Carene	*E. coli*	3 µg/L/OD_{600}	Reiling et al., 2004
	Geraniol	*S. cerevisiae*	989 µg/L/OD_{600}	Oswald et al., 2007
Diterpenoids	Taxadiene	*E. coli*	1300 µg/L	Huang et al., 2001
	Taxadiene	*S. cerevisiae*	1000 µg/L	DeJong et al., 2005
	Taxadien-5α-ol	*S. cerevisiae*	25 µg/L	DeJong et al., 2005
	Casbene	*E. coli*	30 µg/L/OD_{600}	Reiling et al., 2004
Sesquiterpenoids	(+)-δ-Cadinene	*E. coli*	10.3 µg/L	Martin et al., 2001
	5-*Epi*-aristolochene	*E. coli*	0.24 µg/L	Martin et al., 2001
	Vetispiradiene	*E. coli*	6.4 µg/L	Martin et al., 2001
	Amorphadiene	*E. coli*	24000 µg/L [a]	Martin et al., 2003
	Amorphadiene	*E. coli*	480000 µg/L	Newman et al., 2006
	Amorphadiene	*S. cerevisiae*	153000 µg/L	Ro et al., 2006
	Amorphadiene	*S. cerevisiae*	120000 µg/L	Shiba et al., 2007
	Amorphadiene	*S. cerevisiae*	600 µg/L	Lindahl et al., 2006
	Artemisinic acid	*S. cerevisiae*	100000 µg/L	Ro et al., 2006
	Epi-cedrol	*S. cerevisiae*	370 µg/L	Jackson et al., 2003
	5-*Epi*-aristolochene	*S. cerevisiae*	90000 µg/L	Takahashi et al., 2007
	Premnaspirodiene	*S. cerevisiae*	90000 µg/L	Takahashi et al., 2007
	Valencene	*S. cerevisiae*	20000 µg/L	Takahashi et al., 2007
	Capsidiol	*S. cerevisiae*	50000 µg/L	Takahashi et al., 2007
Carotenoids	Lycopene	*E. coli*	25000 µg/g DW	Farmer and Liao, 2001
	Lycopene	*E. coli*	1333 µg/g DW	Matthews and Wurtzel, 2000
	Lycopene	*E. coli*	~ 1000 µg/g DW	Harker and Bramley, 1999
	Lycopene	*E. coli*	22000 µg/L	Kim and Keasling, 2001
	Lycopene	*E. coli*	45000 µg/g DW	Wang et al., 2000
	Lycopene	*E. coli*	1029 µg/g DW	Kajiwara et al., 1997
	Lycopene	*E. coli*	1210 µg/L	Reiling et al., 2004
	Lycopene	*E. coli*	4280 µg/L	Vadali et al., 2005

Lycopene	*E. coli*	102000 µg/L, 22000 µg/g DW	Yoon et al., 2006
Lycopene	*E. coli*	6600 µg/g DW	Alper et al., 2005a
Lycopene	*E. coli*	18000 µg/g DW	Alper et al., 2005b
Lycopene	*E. coli*	220000 µg/L	Alper et al., 2006a
Lycopene	*E. coli*	4700 µg/g DW	Kang et al., 2005
Lycopene	*E. coli*	7700 µg/L	Alper and Stephanopoulos, 2007
Lycopene	*S. cerevisiae*	113 µg/g DW	Yamano et al., 1994
Lycopene	*C. utilis*	758 µg/g DW	Miura et al., 1998a
Lycopene	*C. utilis*	1100 µg/g DW	Miura et al., 1998b
Lycopene	*C. utilis*	7800 µg/g DW	Shimada et al., 1998
Lycopene	*N. crassa*	17.9 µg/g DW	Wang and Keasling, 2002
β-Carotene	*E. coli*	1310 µg/g DW	Kajiwara et al., 1997
β-Carotene	*E. coli*	1533 µg/g DW	Albrecht et al., 1999
β-Carotene	*E. coli*	6000 µg/g DW	Yuan et al., 2006
β-Carotene	*E. coli*	503000 µg/L ,49300 µg/g DW	Yoon et al., 2007
β-Carotene	*E. coli*	390000 µg/L, 4800 µg/L h	Kim et al., 2006
β-Carotene	*S. cerevisiae*	103 µg/g DW	Yamano et al., 1994
β-Carotene	*S. cerevisiae*	5918 µg/g DW	Verwaal et al., 2007
β-Carotene	*C. utilis*	400 µg/g DW	Miura et al., 1998b
β-Carotene	*Z. mobilis*	220 µg/g DW	Misawa et al., 1991
β-Carotene	*A. tumefaciens*	350 µg/g DW	Misawa et al., 1991
Astaxanthin	*E. coli*	1419 µg/g DW	Wang et al., 1999
Astaxanthin	*C. utilis*	400 µg/g DW	Miura et al., 1998b
Zeaxanthin	*E. coli*	289 µg/g DW	Ruther et al., 1997
Zeaxanthin	*E. coli*	592 µg/g DW	Matthews and Wurtzel, 2000
Zeaxanthin	*E. coli*	1570 µg/g DW	Albrecht et al., 1999
Neurosporaxanthin	*N. crassa*	63.4 µg/g DW	Wang and Keasling, 2002

[a:] 112200 µg/L expected if evaporation is taken into account.

1.4.3 Metabolic engineering for heterologous production of novel isoprenoids

Metabolic engineering can also be applied for heterologous microbial production of novel isoprenoids. In the past few years, production of uncommon and non-commercially available carotenoids has drawn much attention because of the increasingly scientific documents indicating their potential applications in preventing cancer and cardiovascular diseases as well as their anti-tumor properties (Cooper et al., 1999). However, production of these complex carotenoids by chemical synthesis is impractical and natural sources are containing only trace amounts of these carotenoids. Hence, microbial production is the method of choice for their commercial production. Expression or combination of carotenogenic genes from different bacteria in *E. coli* was successfully applied for the production of a number of novel hydroxycarotenoids (Albrecht et al., 1997; Albrecht et al., 2000). In another example (Yokoyama et al., 1998), *E. coli* transformants were developed by introducing seven carotenoid biosynthetic genes from *E. uredovora* and *A. aurantiacum* for production of new astaxanthin glucosides. Production of two other uncommon acyclic carotenoids has been achieved in *E. coli* by introducing the *crtC* and *crtD* genes from *Rhodobacter* and *Rubrivivax* (Steiger et al., 2002). Schmidt-Dannert et al. (2000) shuffled phytoene desaturases (encoded by *crtI*) and lycopene cyclases (encoded by *crtY*) from different bacterial species to evolve new enzyme functions and produce a library of carotenoids.

1.5 The choice of microbial host

During the development of novel bioprocesses (or the improvement of existing bioprocesses), the value added element is primarily in the design of efficient cell factories. There are several large research groups and companies focusing on the development of cell factories for novel and/or improved bioprocesses worldwide. Traditionally, biotech processes have been developed based on screening for a microorganism with interesting properties (for example, it produces an interesting compound), whereas in recent years there has been a paradigm shift towards the use of a few well-chosen cell factories. Good examples of this are: 1) the use of a few selected microorganisms to produce a wide range of different enzymes (the Danish company Novozymes has expressed a large number of different enzymes in the filamentous fungus *Aspergillus oryzae*), 2) the use of the penicillin-producing fungus *Penicillium chrysogenum* by the Dutch company DSM for the production of adipoyl-7-aminodeacetoxycephalosporanic acid (adipoyl-7-

ADCA) (Robin et al., 2001), a precursor for the production of semi-synthetic cephalosporins, and 3) the production of the chemical 1,3-propanediol by the American company Dupont by a recombinant *E. coli*, an organism that is already used for the production of many other chemicals, such as phenylalanine. There are several drivers for this development, including:

• Scale-up of bioprocesses can be intensified; when a cell factory has already been used for the production of different products there is extensive empirical knowledge on how a new process based on this cell factory can be scaled-up.

• Fundamental research on the cell factory pays off, as it may impact several different processes. Furthermore, deeper insight into the function of the cell factory is gained through fundamental research, and this enables even wider use of the cell factory for industrial production.

• It may be easier to obtain process (and product) approval when cell factories that are already well implemented are applied.

So far in most of the metabolic engineering studies for microbial production of isoprenoids, *E. coli* or the yeast *S. cerevisiae* have been exploited as the host cells and both of them demonstrated suitability as host platform for the biosynthesis of isoprenoid precursors. These two organisms exhibit several advantages including availability of sequences of their genomes, a plenty of molecular biology tools for the genetic manipulation of cells, and extensive physiological studies on these organisms. Although metabolic engineering tools are available for both organisms, *E. coli* still remains more amenable for genetic modifications. Faster growth of *E. coli* is another advantage compared to *S. cerevisiae* which makes it more economical in industrial fermentation. On the other hand, yeast is generally regarded as safe which is a benefit with regards to social concerns and also makes the approval of both product and process easier and faster.

However, the final choice of microbial host will depend mainly on the type of product and the downstream reactions for the complete biosynthesis of the desired compound. Modifying reactions of the terpene scaffold, such as hydroxylation, lactonization, epoxidation, oxidation and reduction, halogenation, acetylation, and glucosylation are of crucial importance for imparting chemical properties that correlate with biological activities of the final isoprenoid products (Takahashi et al., 2007). Thus, biosynthesis of many of the commercial isoprenoids such as Taxol and artemisinin requires several modifications in the structure of their precursors through one or several of the mentioned downstream reactions. Therefore, for the biosynthesis of plant

isoprenoids, *S. cerevisiae* is considered as a superior host as it provides the biosynthetic machinery for the proper functioning of the downstream modifying enzymes and also has less natural codon bias (Takahashi et al., 2007; Chang and Keasling, 2006).

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Chapter 2

Analytical platform for characterization of sesquiterpene producing yeast strains

2.1 Introduction

Metabolic engineering is a systematic and rational approach for improving cellular phenotypes using directed application of recombinant DNA technology tools for strain improvement (Stephanopulos et al., 1998). Metabolic engineering is a continuous process in which the phenotype of interest is improved in several rounds of modifications until the desired phenotype is obtained. Therefore, metabolic engineering can be considered as a cycle including three main steps (synthesis, analysis, and design) as illustrated in Figure 2.1 (Nielsen, 2001).

Depending on the phenotype of interest, the cycle can be started from different steps. As such, for heterologous production of a metabolite in a microbial host, as it is the case for our study (heterologous production of sesquiterpenes in yeast), it is necessary to start from the synthesis step. However, in order to improve the cell factory for higher productivity or yield of the product, the strains and the pathways involved in the biosynthesis of the product need to be analyzed (Nielsen, 2001). Therefore, developing an analytical platform for characterizing strains and analyzing the products is of crucial importance.

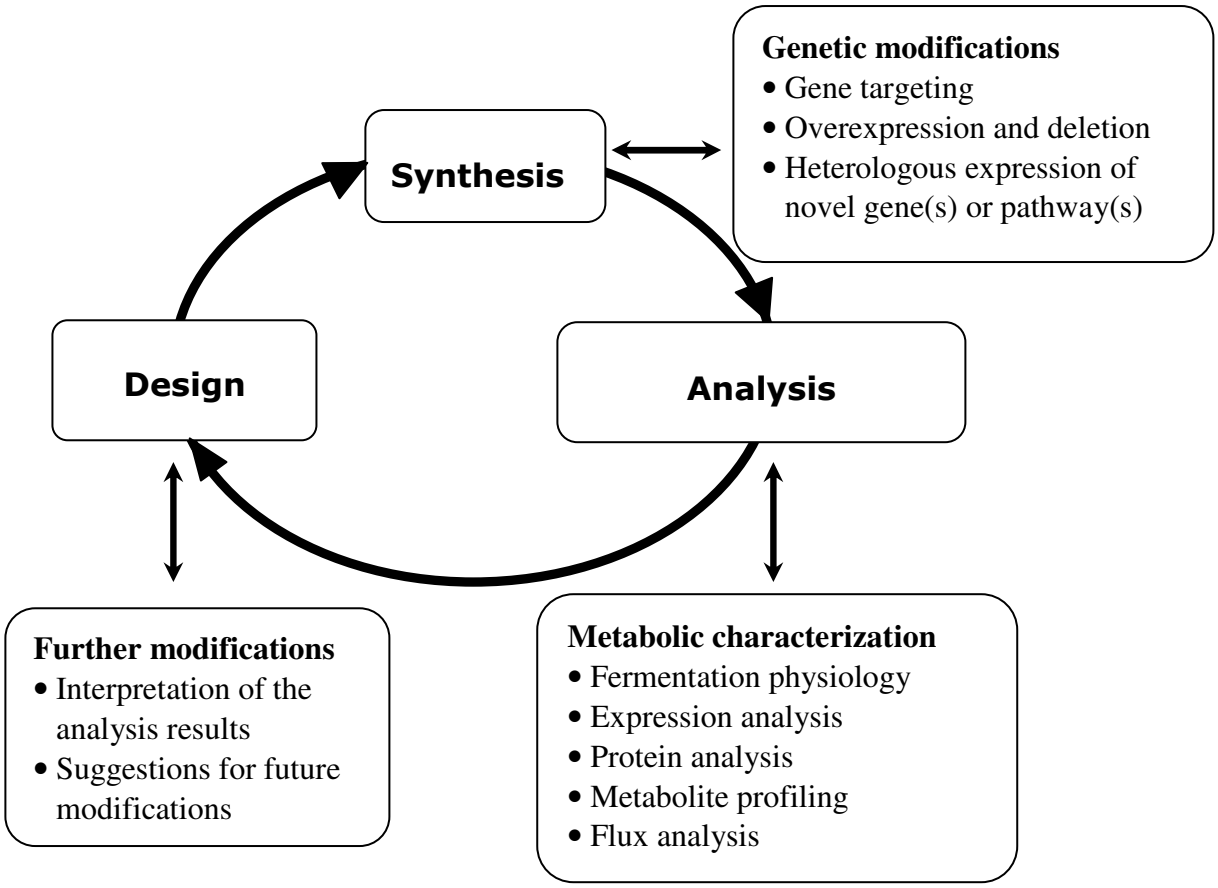

Figure 2.1. The cycle of metabolic engineering (Adopted from Nielsen, 2001)

In this study, we have developed an experimental set up for the characterization of sesquiterpene producing yeast strains. This system is based on a two-phase fermentation where an organic solvent forms the secondary phase and the sesquiterpenes excreted into the medium are immediately collected in the organic layer.

2.2 Materials and methods

2.2.1 Strains and plasmids

The genes encoding valencene synthase (GFTpsD, GenBank accession No. CQ813508) and cubebol synthase (GFTpsC, GenBank accession No. CQ813505) were amplified from cloning vectors containing these genes. The PCR conditions were in accordance with the Expand High Fidelity standard conditions (Roche Applied Science, Mannheim, Germany). Subsequently, the PCR fragments were digested by the restriction enzymes *Eco*RI and *Nhe*I. In parallel, pYX212

plasmid (Ingenius, Wiesbade, Germany) was digested by the same couple of restriction enzymes. The DNA fragments were separated by gel electrophoresis and gel purified using the QIAEX® II Gel extraction kit (Qiagen, Hilden, Germany).

In vitro ligation of the digested plasmid with the digested PCR products was performed as the standard procedure given for T4 DNA ligase (Roche Applied Science, Mannheim, Germany). The resulting ligation mix was used to transform chemically competent *E. coli* cells (DH5α) (Inoue et al, 1990). Transformants were selected on LB medium supplemented with ampicillin (50 mg/L). The constructed plasmids containing GFTpsD and GFTpsC were named pIP031 and pIP032, respectively.

Transformation of the yeast strain CEN.PK113-5D (*MAT*a *MAL2-8*^{c} *SUC2 ura3-52*) with either pIP031 or pIP032 led to the strains YIP-0V-01 and YIP-0C-01, respectively.

2.2.2 Media for shake flasks

Baffled, cotton-stopped, 500 ml Erlenmeyer flasks were used for preparing precultures and also for investigating the effect of different organic solvents on growth. The shake flasks contained 100 ml medium with the following composition: 7.5 g/L $(NH_4)_2SO_4$; 14.4 g/L KH_2PO_4; 0.5 g/L $MgSO_4.7H_2O$; 2 ml/L trace metal solution; and 50 µl/L synperonic antifoam (Sigma, St. Louis, MO). The pH of mineral medium was adjusted to 6.50 by adding 2M NaOH and autoclaved separately from the carbon source solution. Vitamin solution was filter sterilized and aseptically added to the medium after autoclaving at the concentration of 1 ml/L. Shake flasks were incubated in a shaking incubator at 30 ºC and 150 rpm. Organic solvents were added in a 10% (v/v) ratio to the shake flasks.

2.2.3 Media for batch cultivations

A defined minimal medium as described by Verduyn et al. (1992) containing 20 g/L of either glucose or galactose as the sole carbon source was used for all batch fermentations. The medium had the following composition: 5 g/L $(NH_4)_2SO_4$; 3 g/L KH_2PO_4; 0.5 g/L $MgSO_4.7H_2O$; 1 ml/L trace metal solution; 1 ml/L vitamin solution and 50 µl/L synperonic antifoam.

Glucose and galactose were autoclaved separately from the medium and subsequently added to the fermenter, as was the case for the vitamin solution that was added after filter sterilization.

2.2.4 Batch fermentations

Batch fermentations were carried out in well-controlled 5 L in-house manufactured glass bioreactors with a working volume of 4 L. The bioreactors were equipped with two disk-turbine impellers and 4 baffles to ensure proper mixing. The pH was controlled between 4.95 and 5.05 by automatic addition of 2M NaOH. The temperature was kept constant at 30 ºC. The air flow was 4 L/min (1 vvm) and was sterilized by filtration and the off gas passed through a condenser.

2.2.5 Liquid-liquid extraction (LLE)

Valencene and cubebol from culture aliquots of 1 ml were extracted with an equal volume of hexane. Extraction was performed in glass vials and mixture was vortexed for 2 min. After equilibration and phase separation, the hexane extract was analyzed using GC-MS. The hexane extract was concentrated under a gentle nitrogen stream prior to GC-MS analysis.

2.2.6 Collection of sesquiterpenes from off-gas using adsorbent tubes

Sesquiterpenes from the fermenter off-gas were collected either in glass tubes containing coconut charcoal as adsorbent (SKC, 8 × 110 mm, 2-section, 200/400 mg sorbent) or in stainless steel Perkin Elmer (PE) tubes filled with 200 mg of Tenax-TA adsorbent material. PE tubes were conditioned before use by heating to 300 ºC for 1 h while being purged with approximately 10 ml/min nitrogen (purity > 99.999%).

In order to collect sesquiterpenes carried with the off-gas, adsorbent tubes were connected to the outlet gas tube from fermenter (with both ends open). A gas drier tube (W.A. Hammond Drierite, Xenia, OH) was placed before the adsorbent tubes to remove trace humidity in the outlet air. Adsorbent tubes were replaced with new tubes and analyzed every two hours. Thus, the total amounts of sesquiterpenes carried with the off-gas were the sum of all analyses.

Sesquiterpenes trapped on the SKC tubes were extracted with 2 ml hexane and allowed to stand with occasional shaking for 30 min. The sesquiterpenes in the hexane extract were detected and analyzed with GC-MS.

Sesquiterpenes trapped on PE tubes were thermally desorbed on a Perkin-Elmer ATD 400 coupled to a Hewlett-Packard 5890 gas chromatograph further coupled to a HP 5972 mass spectrometer. Sesquiterpenes were separated on a VB-5 ValcoBond capillary column (SIS, Ringoes, NJ) using He as carrier gas. Initial pressure was 13 psi, and the He flow rate was 1

ml/min. The system was run in a split (75:1) mode and the injection temperature was set to 250 ºC. The initial oven temperature was 35 ºC. After 1 min, the oven temperature was raised to 175 ºC at the ramp of 4 ºC/min and then to 260 ºC at the ramp of 10 ºC/min. Mass spectra were recorded at 70 eV, scanning from m/z 33 to 330.

2.2.7 Analysis of sesquiterpenes by solid phase microextraction (SPME) method

Identification of sesquiterpenes produced by the yeast strains transformed with heterologous sesquiterpene synthases was performed by extracting volatile compounds from the head-space of the samples on 100 µm polydimethyl siloxane (PDMS) fibers (Supelco, Bellefonte, PA, USA). Fibers were conditioned according to the suppliers' instruction. Two ml samples from the culture media were immediately frozen and stored in 4 ml screw cap glass vials at -20 ºC until analysis. The contents of vials were thawed on ice and allowed to equilibrate at room temperature. Extraction was performed for 25 min at 60 ºC while mixing the sample with a small magnet. After extraction, the analytes were thermally desorbed at 250 ºC from SPME fiber into the injector of gas chromatogram in the splitless mode. The oven temperature was held initially at 45 ºC for 1 min, then raised to 130 ºC by a ramp of 10 ºC/min followed by a ramp of 3 ºC/min to 160 ºC. The oven temperature was further raised to 250 ºC at 10 ºC/min and maintained at this temperature for 5 min to equilibrate.

2.2.8 Analysis of sesquiterpenes in the organic phase

Samples from organic layer (obtained either from LLE of culture aliquots or from two-phase fermentations) were analyzed by GC-MS to determine the level of sesquiterpenes. GC-MS analyses were run on a Thermo Finnigan Focus GC coupled to a Focus DSQ quadrupole mass spectrometer. Analytes from 1 µl samples were separated on a SLB-5ms capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Supelco, Bellefonte, PA, USA) using He as carrier gas at the flow rate of 1.2 ml/min. A split/splitless injector was used in the splitless mode. The initial oven temperature was 80 ºC (50 ºC for samples obtained from LLE), and injector temperature was 250 ºC. After 1 min, the oven temperature was increased to 120 ºC at the rate of 10 ºC/min and subsequently increased to 160 ºC at the rate of 3 ºC/min. The oven temperature was finally increased to 270 ºC at the rate of 10 ºC/min and held for 5 min at this temperature. When necessary, to increase the sensitivity, the MS was run in selected ion monitoring (SIM) mode

using ions of m/z 133, 161, and 204 (for valencene) and 161, 207, and 222 (for cubebol) which represent the molecular ions and two abundant ions for these sesquiterpenes.
Quantification of compounds was carried out using standard curves generated after each analysis run.

2.2.9 Analysis of glucose and ethanol

To determine the concentration of glucose and ethanol in the culture media, 2 ml samples were withdrawn from the fermenter and immediately filtered through a 0.45 µm pore-size cellulose acetate filter (Sartorius AG, Göttingen, Germany). The filtrate was stored at -20 ºC until HPLC analysis. Glucose and ethanol concentrations were determined in a Waters 717 plus Autosampler HPLC system equipped with a Bio-Rad Aminex HPX-87H reverse phase column (Biorad, Hercules, CA) at 60 ºC using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min. Glucose and ethanol were detected refractometrically (Waters 410 Differential Refractometer Detector, Millipore Corp., Milford, MA).

2.3 Results

2.3.1 Extraction of sesquiterpenes by LLE

Sesquiterpenes are very hydrophobic compounds and therefore extraction of the excreted sesquiterpenes from the culture by an organic solvent like hexane seemed to be the method of choice. However, it was very difficult to detect peaks for valencene and cubebol in the hexane extracts and the extracts needed to be upconcentrated several-fold. Low concentration of sesquiterpenes produced by the yeast strains could be due to poor expression of plant sesquiterpene synthases (Martin et al., 2001).

2.3.2 SPME is a more powerful method for extraction and concentration of sesquiterpenes

The low concentration of sesquiterpenes in the culture made it necessary to use more sensitive techniques for their analysis. SPME, introduced in the early 1990s (Arthur and Pawliszyn, 1990; Zhang and Pawliszyn, 1993), was used as a powerful alternative method to the traditional LLE.
In this study, we used 100 µm PDMS fibers for extraction of sesquiterpenes from the culture. The optimum extraction temperature and exposure time were found by extraction of valencene and cubebol standards spiked into water at different time periods and temperatures. Thus, the

optimum temperature and exposure time were found to be 60 ºC and 25 min, respectively. Addition of salt did not improve the extraction efficiency as these two compounds are already highly hydrophobic.

Extraction of sesquiterpenes from the fermentation medium using SPME and their analysis with GC-MS gave rise to very nice peaks and large peak areas compared to those obtained with the LLE method proving the capability and sensitivity of the SPME method for sample preparation. However, we noticed that the sesquiterpene concentration in the fermentation medium increased as cells grew and reached to a maximum but afterwards a drastic drop in the concentration was observed (Figure 2.2). This rapid decrease in the sesquiterpene concentration could be due to loss of these compounds through the off-gas. Martin et al. (2003) also observed loss of amorphadiene to the headspace in cultures inoculated with engineered amorphadiene producing *E. coli* strains. To assess if the loss of sesquiterpenes was a consequence of evaporation into the gas phase, a fermenter containing distilled water was spiked with standard valencene and the valencene concentration in the water phase was measured as a function of time in the presence or absence of agitation and aeration using SPME-GC-MS (Figure 2.3). The results clearly show that valencene has a high partitioning into the gas phase which is greatly accelerated by agitation and aeration.

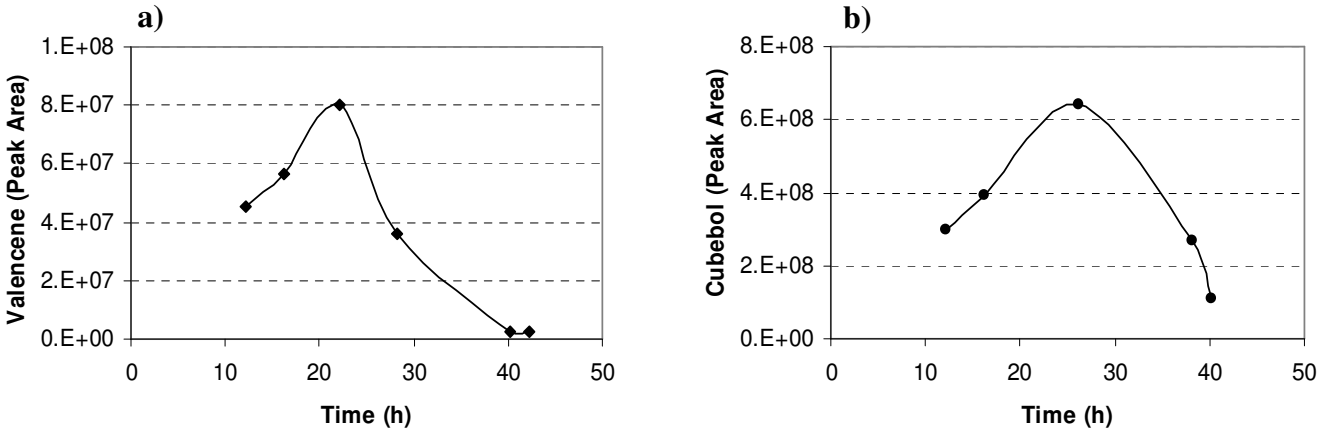

Figure 2.2. Sesquiterpene concentrations measured by SPME-GC-MS as a function of time for (a) YIP-0V-01 and (b) YIP-0C-01
Cells were grown on 4 L ml minimal medium containing 20 g/L glucose in 5 L batch fermenters.

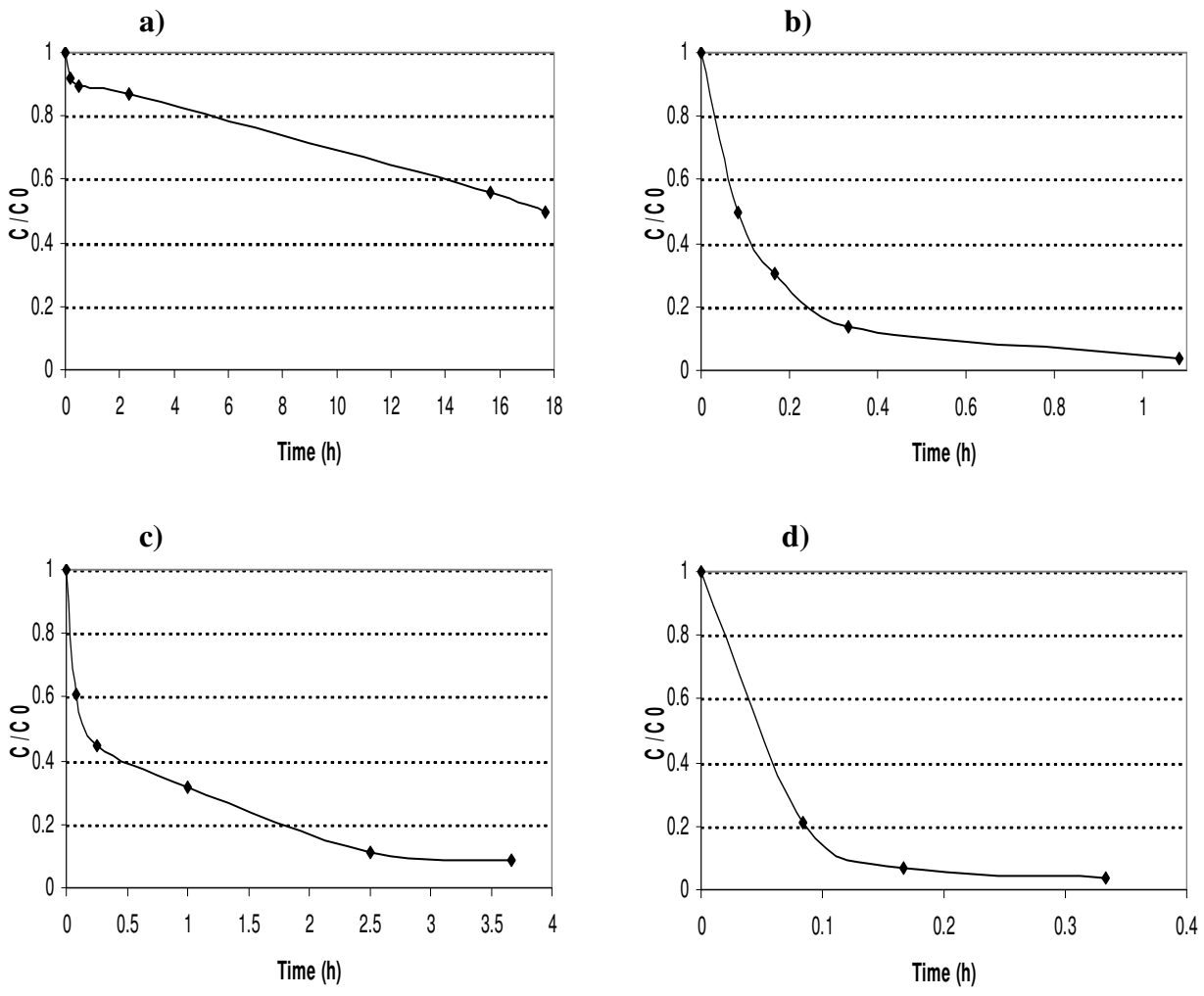

Figure 2.3. The ratio of valencene concentration at different time points to its initial concentration in the fermenters containing distilled water spiked with standard valencene (a) No aeration and no agitation (b) No aeration, agitation: 700 rpm (c) Aeration: 1 vvm, no agitation (d) Aeration: 1 vvm, Agitation: 700 rpm

2.3.3 Trapping sesquiterpenes from the off-gas

Substantial loss of valencene and cubebol through the off-gas is a major problem for characterization of engineered sesquiterpene producing yeast strains. Characterization of strains for sesquiterpene production requires measurement of total produced sesquiterpenes and therefore the lost sesquiterpenes in the off-gas need to be somehow collected and analyzed. We examined different methods for collecting sesquiterpenes from the off-gas.

The fermenter off-gas was first passed through the PE tubes filled with Tenax-TA adsorbent. By desorbing the trapped compounds using an automatic thermal desorber coupled to a GC-MS, it

was possible to detect the sesquiterpenes peaks. However, quantification of the lost compounds required making suitable standard curves which was very difficult as sampling was done from gas phase and no solvent was used for desorbing the collected compounds. Therefore we tested the glass tubes filled with coconut charcoal in two separate zones to ensure complete collection of all compounds in the gas phase. By extracting the desorbed compounds from each zone into hexane and making standard curve it was possible to quantify the lost sesquiterpenes. However, the collected sesquiterpenes on the adsorbent was only a fraction of the lost compounds and the adsorbent was not able to collect all the sesquiterpenes. This could be because the trace humidity in the air flow made the adsorbent matrix wet and sticky and prevented total adsorption onto the adsorbent. Although this problem was circumvented by using gas drier tubes before the adsorbent tubes, there was a risk that the sesquiterpenes were retained on the drying material. Hence, instead of passing the off-gas through adsorbent tubes, we decided to pass it through an organic solvent and analyze the organic solvent for the presence of sesquiterpenes. The organic solvent was upconcentrated and analyzed by GC-MS. This method also allowed detection and quantification of the sesquiterpenes in the gas phase but again the trapped sesquiterpene in the organic solvent was only a fraction of the total lost sesquiterpene. Since sesquiterpenes are very hydrophobic and they have to pass through the condenser, outlet air filter and silicon tubes before entering the adsorbent tubes or organic solvent, we speculated that most of the sesquiterpenes were bound to the air filter, silicon tubes or other parts of the fermenter before getting to the collection units.

2.3.4 *In situ* separation of sesquiterpenes from the culture media

Considering all the practical problems faced for collection of the lost sesquiterpenes from the off-gas, the best solution for quantification of the produced sesquiterpenes would be to use *in situ* separation from the fermentation medium before they are evaporated into the gas phase. This can be achieved by two different methods: addition of adsorbent resins directly to the culture for separation of hydrophobic compounds or trapping the sesquiterpenes into a secondary organic phase which is added to the fermenter.

Synthetic adsorbent resins are highly porous spherical crosslinked polymer particles with no ion-exchange or functional groups that adsorb organic compounds on the surface due to hydrophobic interactions. These resins were successfully used for separation of pigments (Bae et al., 2001)

and epi-cedrol (Jackson et al., 2003) from culture media. However, desorption of compounds from resins is time consuming and involves several steps of extraction and concentration before they can be analyzed. Furthermore, utilizing these resins only allows measurement of final sesquiterpene titer and it is not possible to follow the concentration profile throughout a fermentation process. *In situ* separation of sesquiterpenes can alternatively be achieved using a two-phase fermentation where the secondary phase is an organic phase that traps the released sesquiterpenes quickly. Selection of an appropriate solvent is the key driver for successful operation of a two-phase fermentation. We first identified three different organic solvents (diisononyl phthalate, oleyl alcohol, and dodecane) which seemed to have suitable characteristics (biocompatibility, immiscibility, and low volatility) and investigated their applicability in two-phase fermentations in shake flasks with *S. cerevisiae*. None of these solvents had significantly detrimental effects on the specific growth rate (Figure 2.4). However, phase separation was faster and clearer when dodecane was the organic solvent, and this solvent was therefore chosen for the subsequent experiments. The effect of dodecane on the growth was further examined in a 5 L batch fermenter, and it was found not to have any negative effects on cell growth (Figure 2.5).

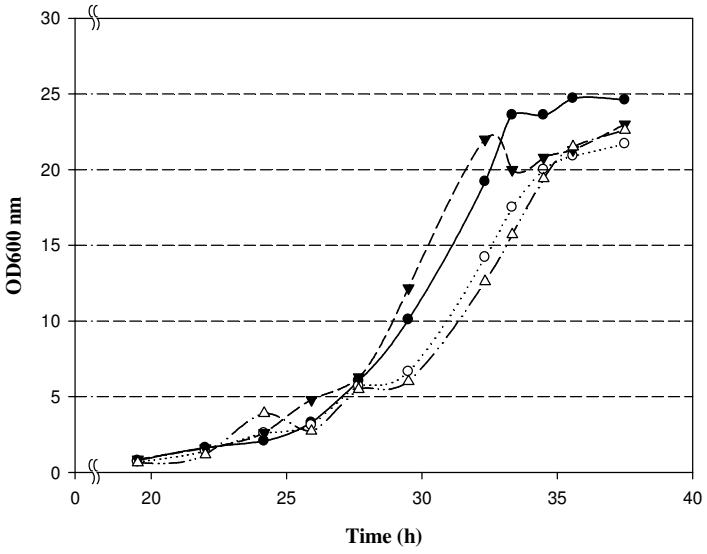

Figure 2.4. Growth pattern of YIP-0V-01 strain in the presence of 10% (v/v) different organic solvents; No solvent: ●; Diisononyl phthalate: ▼; Dodecane: ○; Oleyl alcohol: △
Cells were cultivated in shake flasks containing 100 ml minimal medium and 15 g/L glucose. Organic solvent was added aseptically at OD=1 ± 0.1.

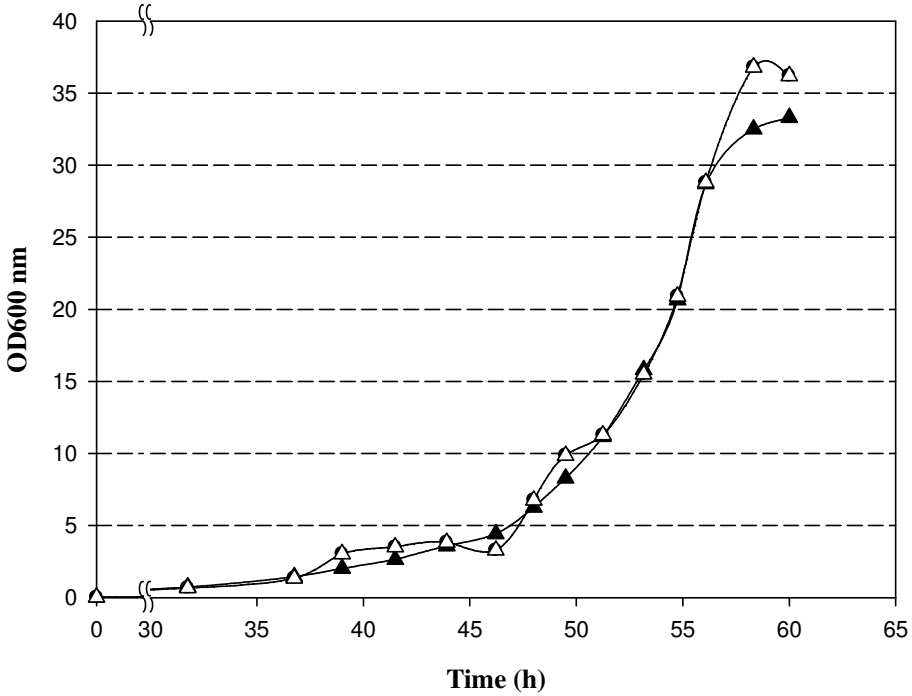

Figure 2.5. Growth pattern of YIP-0V-01 with (△) and without (▲) dodecane.
Cells were grown on 4 L ml minimal medium containing 20 g/L galactose in 5 L batch fermenters. 300 ml dodecane was added aseptically at OD=1 ± 0.1.

YIP-0C-01 strain was then characterized in two-phase 5 L batch fermenters containing 4 L minimal medium and 20 g/L glucose. Samples were taken from the organic phase and culture medium at different times and were analyzed for sesquiterpene, biomass, glucose and ethanol. Time-course analysis (Figure 2.6) showed collectively accumulation of sesquiterpenes in the dodecane phase as cells were growing and proved the potential of this system for capturing the hydrophobic sesquiterpenes. No drop in sesquiterpene concentration was observed even after depletion of all the carbon sources. At the end of fermentation a sample from the aqueous phase was analyzed by SPME-GC-MS to check the presence of sesquiterpenes in the aqueous phase. However no sesquiterpenes were detectable reflecting the high partition coefficient of these compounds into the dodecane phase. It was therefore concluded that two-phase fermentations using dodecane as organic phase presents a suitable platform for characterization of sesquiterpene producing yeast strains.

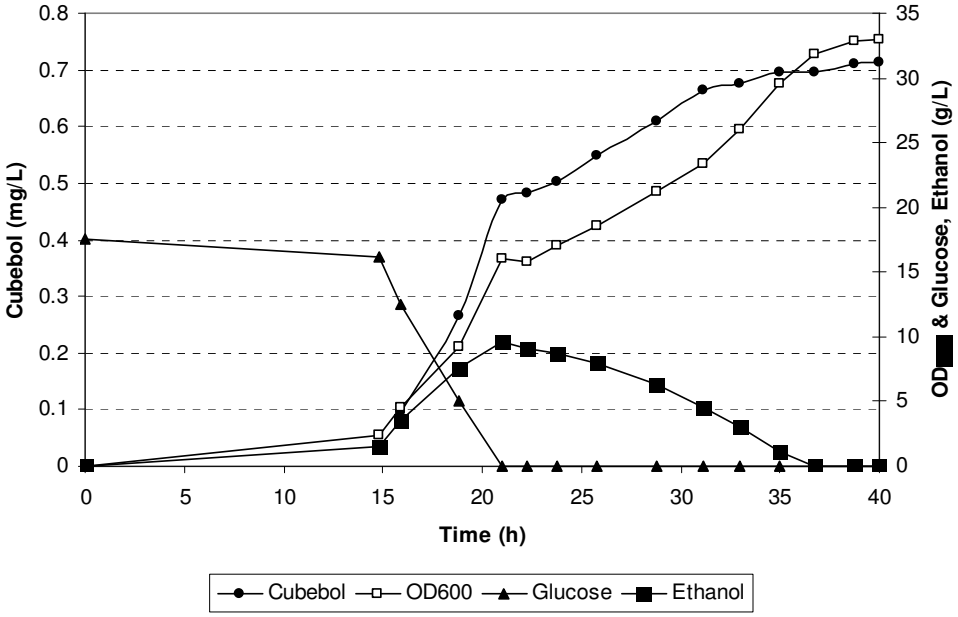

Figure 2.6. Cubebol, glucose, ethanol and OD_{600} as a function of time for YIP-0C-01 strain. Cells were grown on 4 L ml minimal medium containing 20 g/L glucose in 5 L batch fermenters. 300 ml dodecane was added aseptically at OD=1 ± 0.1.

2.4 Discussion

Towards establishment of yeast as a platform for production of sesquiterpenes, yeast strains were transformed with valencene synthase and cubebol synthase and were shown to produce these two sesquiterpenes by their extraction from culture media with hexane followed by GC-MS analysis. However, the sesquiterpene concentration in the culture was very low and the hexane extract had to be upconcentrated many folds. SPME is a solvent-free sample preparation technique in which a fine silica fiber coated with a thin layer of a selective coating is exposed either into the headspace above the sample or directly into the sample to extract and concentrate organic compounds. After equilibration has reached between the fiber and analytes, the SPME fiber is readily introduced into the injection port of the GC instrument for thermal desorption. Thus, SPME integrates extraction, concentration and sample introduction steps into a single step. Although SPME is not based on exhaustive extraction of compounds, all the extracted analytes are transferred to the analytical instrument which improves the sensitivity compared to the traditional LLE method where only a small portion of the analyte is injected. Extraction of sesquiterpenes from fermentation media by SPME followed by GC-MS presented a suitable

method for detection of even trace amounts of sesquiterpenes. Nonetheless, this method was not chosen for characterization of strains since a dramatic decrease in the sesquiterpene concentration was observed during fermentation. Analysis of water spiked with standard valencene in a fermenter confirmed the rapid loss of valencene through the gas phase. However, with the high boiling point of valencene which is 274 ºC at atmospheric pressure the question arises how valencene can behave as a volatile compound. Since valencene is highly hydrophobic, it is sparingly soluble in water and therefore its partial pressure in the gas phase is calculated from Henry's law equation for dilute solutions:

$$p^*_{val} = H_{val} c_{val}$$

where p^*_{val} is the partial pressure of valencene in the gas phase, H_{val} is Henry's law constant for valencene, and c_{val} is the valencene concentration in the aqueous phase.

The partial pressure of valencene in the gas phase is therefore highly dependent on its Henry's law constant. This constant has not been reported for valencene in the literature but isoprene and monoterpenes have very high Henry's law constant (Niinemets and Reichstein, 2003). For instance, the reported values for isoprene and α-pinene are 7780 and 10840 Pa m^3 mol^{-1}, respectively, whereas it is only 3.88 Pa m^3 mol^{-1} for acetone (boiling point 56.3 ºC). Humulene which is a sesquiterpene has a Henry's law constant of 16500 Pa m^3 mol^{-1} (Jürgens et al., 2007). These large Henry's law constants indicate big deviations from ideal solution behavior and presence of a high partial pressure in the gas phase for these compounds despite their high boiling points and low concentrations in the water phase. This could explain why valencene molecules partition into the gas phase at a high rate. It is noteworthy that this partitioning is only due to interaction of valencene with water molecules which makes their mixture a very non-ideal solution. Therefore volatility is not the correct word to describe the reason for loss of valencene from the water phase, even though valencene (Lücker et al., 2004) and generally sesquiterpenes (Tamogami et al., 2007) are commonly referred to as volatiles in the literature. This mechanism of moving valencene molecules from the liquid phase to the gas phase holds true if the valencene concentration in water is below its solubility limit. If valencene concentration exceeds this limit then the extra valencene forms a new phase and other mechanisms could also contribute to its loss to the gas phase as well. In this case, if there is no mixing and aeration, since valencene has lower density than water, it forms a layer on top of the mixture and evaporation mainly occurs

from this pure layer of valencene which would be very low compared to its loss when it is dissolved in water. If the mixture is disturbed by aeration or agitation, besides evaporation of valencene from solution, the undissolved valencene which is now dispersed in water would also be stripped into the gas phase in the form of aerosols.

To evaluate total sesquiterpene production by these strains, we next sought to analyze the loss of sesquiterpenes in the gas phase by trapping them in either adsorbent tubes (Tenax-TA or coconut charcoal) or in an organic solvent. Even though we were able to detect sesquiterpenes by both methods, practical problems such as difficulties in making a good standard curve (for the case of PE tubes containing Tenax-TA), adhesion of adsorbents because of humidity in the outlet air, and adsorption of sesquiterpenes onto different parts of the fermenter before entering the collection unit, none of these methods were chosen for further experiments.

In a further attempt, we tested *in situ* separation of sesquiterpenes in a two-phase fermentation and showed that this method can be used for analyzing the total produced sesquiterpenes and therefore is suitable for characterization of strains. Two-phase fermentation has been successfully used for *in situ* separation of products (Frenz et al., 1989; Sim et al., 2001) and also for controlled substrate delivery for biodegradation of toxic substrates where high concentration of substrate could kill or inhibit the microorganisms (Collins and Daugulis, 1999). All three tested solvents were appropriate for using in a two-phase fermentation with yeast but dodecane was chosen as its phase separation was faster and more distinct and also because it has been used as an oxygen vector for improving oxygen transfer in fermenters (Rols et al., 1990). Two-phase fermentation using dodecane as the organic phase demonstrated a suitable platform for characterization of sesquiterpene producing yeast strains and enabled collection and analysis of all the produced sesquiterpenes. No loss of sesquiterpenes was observed even after depletion of all the carbon sources and cessation of growth. This proposes that the loss of sesquiterpenes did not occur because of their degradation in the culture medium. Moreover, constant level of sesquiterpenes at the end of fermentation suggests a low Henry's law constant for these compounds in dodecane and further proves that these compounds are not volatile and their loss through the gas phase was mainly due to their interaction with water molecules at low concentration.

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Chapter 3

Effect of *ERG9* repression on sesquiterpene biosynthesis in yeast

3.1 Introduction

Tens of thousands of metabolites including isoprenoids, alkaloids, phenylpropanoids and all allied phenylic compounds are synthesized by plants (Croteau et al., 2000). The isoprenoids with more than 40,000 described examples (Withers and Keasling, 2007) comprise the largest and most structurally diverse group of plant metabolites. Sesquiterpenes are the most diverse class of isoprenoids with more than 300 identified carbon skeletons (Cane, 1999) and more than 7,000 characterized compounds (Connolly and Hill, 1991). Sesquiterpenes play numerous important ecological roles in plants e.g. in pollinator attraction, as phytoalexins and antifeedants (Harborne, 1991).

From an industrial point of view, sesquiterpenes are interesting compounds because of their potent anticancer, antitumor, cytotoxic, antiviral and antibiotic properties as well as their characteristic flavors and aromas. Most sesquiterpenes are constituents of plant essential oils, but extraction from plants is not a suitable method for large scale production of many sesquiterpenes. Furthermore, using plants as resources for industrial production of sesquiterpenes include slow growth, varying composition and concentration depending on the geographical position and

climate conditions. There is therefore much interest in using microorganisms as cell factories for the production of isoprenoids. The intracellular pools of isoprenoid precursors in microorganisms appear, to be however, not enough to provide high level production. It may therefore be necessary to deregulate the pathways involved in the biosynthesis of isoprenoid precursors in order to improve production (Kim and Keasling, 2001, Martin et al., 2003; Pitera et al., 2007). In the last decade numerous studies have been conducted with regard to engineer microorganisms for the production of different isoprenoids (for review see Maury et al., 2005). In most studies, *E. coli* has been used as the microbial platform for metabolic engineering of isoprenoid production (Alper et al., 2005; Carter et al., 2003; Martin et al., 2001). However, in some cases the mevalonate pathway in yeasts has been deregulated to improve the biosynthesis of different isoprenoids (DeJong et al., 2006; Jackson et al., 2003; Miura et al., 1998a; Miura et al., 1998b; Shimada et al., 1998; Yamano et al., 1994; Verwaal et al., 2007; Takahashi et al., 2007; Ro et al., 2006). In *S. cerevisiae* the mevalonate pathway is the only pathway involved in the biosynthesis of isoprenoid precursors. This pathway leads to the formation of ergosterol as the end product of the pathway, the content of which in *S. cerevisiae* can reach 5% of the dry weight (Lamačka and Šajbidor, 1997). Yeast therefore has a high inherent capacity for the biosynthesis of isoprenoid precursors that may be directed to the production of heterologous compounds.

In this study, we chose *S. cerevisiae* as a host cell for heterologous production of three different plant sesquiterpenes namely valencene, cubebol and patchoulol. These three compounds are derived directly in a single enzymatic step from FPP, the biological precursor for all sesquiterpenes. In the case of ergosterol, FPP is converted to squalene by squalene synthase, which is encoded by the *ERG9* gene. In order to increase the level of FPP and direct flux towards sesquiterpenes an obvious strategy is to attenuate the expression of the *ERG9* gene. Since ergosterol is vital for yeast growth and yeast is unable to assimilate exogenous ergosterol during aerobic growth conditions, this gene can not be completely deleted. We therefore controlled the *ERG9* expression by using the *MET3* promoter (Gardner and Hampton, 1999; Mountain et al., 1991) to drive the expression of *ERG9*. This strategy has been successfully reported to increase 2-fold the final titer of amorphadiene in an engineered yeast strain (Ro et al., 2006).

Characterization of engineered yeast strains was performed in two-phase batch fermenters using dodecane as the secondary phase. This allowed *in situ* separation of released sesquiterpenes before they are lost through the off-gas.

3.2 Materials and methods

3.2.1 Cloning of *MET3* promoter

The *MET3* promoter was amplified from genomic DNA of *S. cerevisiae* CEN.PK 113-7D using the primers Met3pf_bis and Met3pr_bis (Table 3.1). The PCR conditions were in accordance with the Expand High Fidelity standard conditions (Roche Applied Science, Mannheim, Germany). Subsequently, the PCR fragments were digested by the restriction enzymes *Spe*I and *Sac*II. In parallel, pUG6 plasmid (Güldener et al., 1996) was digested by the same couple of restriction enzymes. The DNA fragments were separated by gel electrophoresis and gel purified using the QIAEX® II Gel extraction kit (Qiagen, Hilden, Germany).

In vitro ligation of the digested plasmid with the digested PCR products was performed as the standard procedure given for T4 DNA ligase (Roche Applied Science, Mannheim, Germany). The resulting ligation mix was used to transform chemically competent *E. coli* cells (DH5α) (Inoue et al., 1990). Transformants were selected on LB medium supplemented with ampicillin (50 mg/L). The plasmid obtained was named pIP007.

3.2.2 *ERG9* promoter replacement by *MET3* promoter

In order to replace the *ERG9* promoter by the *MET3* promoter, fusion PCR and a bipartite gene targeting method (Erdeniz et al., 1997) were applied. Four fragments were separately amplified before fusing them together in pairs. First, two fragments containing the *MET3* promoter and the KanMX selection cassette were amplified from pIP007 in two separate, but overlapping, fragments using the two couples of primers (Table 3.1): pUG6met3f/ KanMXr (Fragment A) and KanMXf/ pUG6met3r (Fragment B). Furthermore, 500 bp upstream of the *ERG9* promoter in the genome of *S. cerevisiae* were amplified using the primers ERG9_up_f and ERG9_up_r (Fragment C). The first 500 bp of the *ERG9* ORF were as well amplified using the primers ERG9f and ERG9r (Fragment D).

The resulting four PCR fragments were gel purified using the High Pure PCR Product Purification kit (Roche Applied Science) and subsequently fused together in pairs using fusion

PCR. Fused fragments A and C were obtained after a fusion PCR using the primers ERG9_up_f and KanMXr while fused fragments B and D were obtained after a fusion PCR using the primers KanMXf and ERG9r (Table 3.1). The two final fusion PCR fragments were gel purified with the High Pure PCR Product Purification kit (Roche Applied Science).

3.2.3 Strain construction

Strain *S. cerevisiae* YIP-0V-01, obtained by transformation of strain YIP-00-03 with the plasmid pIP031 (plasmid obtained by cloning GFTpsD in pYX212 using *EcoR*I and *Nhe*I restriction sites) was transformed with the obtained fusion PCR fragments to result in strain YIP-MV-02.

Strain YIP-M0-04 was obtained after exclusion of plasmid pIP031 from strain YIP-MV-02 by selection on plates containing 5-fluoroorotic acid (5-FOA). Strains YIP-MC-02, YIP-MV-01 and YIP-MP-01 were obtained after transformation of strain YIP-M0-04 with either pIP032 (obtained by cloning GFTpsC into pYX212 using *EcoR*I and *Nhe*I restriction sites), pIP027 (obtained by cloning GFTpsD into pESC-URA using *Age*I and *Sac*II restriction sites) or pIP029 (obtained by cloning PatTps177 into pESC-URA using *BamH*I and *Xho*I restriction sites), respectively.

Transformation of strain YIP-00-03 with either pIP032, pIP013 (obtained by cloning of GFTpsC into pESC-TRP by gap repair using the two primers Gap_Trp_f and Gap_Trp_r (Table 3.1)), pIP027 or pIP029 led to the strains YIP-0C-01, YIP-0C-02, YIP-0V-02 and YIP-0P-02, respectively. Table 3.2 demonstrates the strains used in this study.

Sequences of the genes GFTpsD (valencene synthase), GFTpsC (cubebol synthase) and PatTps177 (patchoulol synthase) can be obtained from GenBank: accession numbers CQ813508, CQ813505 and AY508730, respectively.

3.2.4 Media for shake flasks

Baffled, cotton-stopped, 500 ml Erlenmeyer flasks were used for preparing precultures and also for preparing samples for ergosterol measurement. The shake flasks were containing 100 ml medium with the following compositions: 7.5 g/L $(NH_4)_2SO_4$; 14.4 g/L KH_2PO_4; 0.5 g/L $MgSO_4.7H_2O$; 2 ml/L trace metal solution; 1 ml/L vitamin solution and 50 µl/L synperonic antifoam (Sigma® A-5551). The pH of mineral medium was adjusted to 6.50 by adding 2M NaOH and autoclaved separately from carbon source solution. Vitamin solution was filter

sterilized and aseptically added to the medium after autoclavation. Shake flasks were incubated in a shaking incubator at 30 ºC and 150 rpm.

3.2.5 Media for batch cultivations

A defined minimal medium as described by Verduyn et al. (1992) containing 20 g/L of either glucose or galactose as the sole carbon source was used for all batch fermentations. The media had the following compositions: 5 g/L $(NH_4)_2SO_4$; 3 g/L KH_2PO_4; 0.5 g/L $MgSO_4.7H_2O$; 1 ml/L trace metal solution; 1 ml/L vitamin solution and 50 µl/L synperonic antifoam. Expression of *ERG9* was repressed by supplementing media with 2 mM filter sterilized methionine.

Glucose and galactose were autoclaved separately from the medium and subsequently added to the fermenter, as was the case for the vitamin solution that was added after filter sterilization.

Table 3.1. Primers used in this study

Primer	Sequence
Met3pf_bis	ggactagtcctTGGTATAAGGTGAGGGGGTCCACAG
Met3pr_bis	tccccgcgggaGAATACCACCGTGAGGAGCAGGCATG
pUG6met3f	gatccccgggaattgccatgACGCTGCAGGTCGACAACCC
pUG6met3r	caatgccaattgtaatagcttcccatGTTAATTATACTTTATTCTTGTTATTATTATAC
KanMXf	CTATCGATTGTATGGGAAGCCCG
KanMXr	CCATGAGTGACGACTGAATCCGG
ERG9_up_f	AGCCTCAGTACGCTGGTACCCG
ERG9_up_r	catggcaattcccggggatcTGGGCTATGAAATGTACTGAGTCAG
ERG9f	ATGGGAAAGCTATTACAATTGGCATTG
ERG9r	GTCGTAGTCGTGGACGGTTTGC
Gap_Trp_f	ATACTTTAACGTCAAGGAGAAAAAACCCCGGATCCCGTTatggcacttcaagattcaga
Gap_Trp_r	TCTTCTTCGGAAATCAACTTCTGTTCCATGTCGACGCTtcaaaagggaacaggcttct

Table 3.2. Strains used in this study

Strain	Genotype	Plasmid	Plasmid description
YIP-00-03 (CEN.PK113-5D)	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	none	
YIP-0C-01	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP032	pYX212 2µ *URA3* P_{TPI1}-GFTpSC
YIP-0C-02	*MAT***a** *MAL2-8*^{c} *SUC2 trp1-289*	pIP013	pESC-TRP 2µ *TRP1* P_{GAL1}-GFTpSC
YIP-0P-02	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52,*	pIP029	pESC-URA 2µ *URA3* P_{GAL1}- PatTps177
YIP-0V-01	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP031	pYX212 2µ *URA3* P_{TPI1}-GFTpSD
YIP-0V-02	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP027	pESC-URA 2µ *URA3* P_{GAL1}- GFTpSD
YIP-M0-04	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	none	
YIP-MC-02	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	pIP032	pYX212 2µ *URA3* P_{TPI1}-GFTpSC
YIP-MP-01	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	pIP029	pESC-URA 2µ *URA3* P_{GAL1}- PatTps177
YIP-MV-01	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	pIP027	pESC-URA 2µ *URA3* P_{GAL1}- GFTpSD
YIP-MV-02	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	pIP031	pYX212 2µ *URA3* P_{TPI1}-GFTpSD

3.2.6 Batch fermentations

Batch fermentations were carried out in well-controlled 5 L in-house manufactured glass bioreactors with a working volume of 4 L. The bioreactors were equipped with two disk-turbine impellers and 4 baffles to ensure proper mixing. The pH was controlled between 4.95 and 5.05 by automatic addition of 2M NaOH. The temperature was kept constant at 30 ºC. The air flow was 4 L/min (1 vvm) and was sterilized by filtration and the off gas passed through a condenser. Carbon dioxide and oxygen concentrations in the off-gas were determined by a Brüel & Kjær acoustic gas analyzer (Brüel & Kjær, Nærum, Denmark). Batch fermenters were inoculated to an initial OD_{600} of 0.02 from a liquid preculture. Three hundred milliliters of dodecane was added aseptically to the media at OD_{600} 0f 1 ± 0.1.

3.2.7 OD and dry weight determinations

The OD of samples was determined at 600 nm by using a Hitachi U-1100 spectrophotometer. Dry weight measurement was achieved using 0.45 µm pore-size nitrocellulose filters (Sartorius AG, Göttingen, Germany) according to the method described by Dynesen et al. (1998).

3.2.8 Extraction of ergosterol

Baffled, cotton-stopped, shake flasks containing 100 ml minimal medium and 20 g/L glucose as carbon source were inoculated with overnight precultures to an initial OD_{600} of 0.02 and incubated for 24 h in a shaking incubator at 30 ºC and 150 rpm. A certain volume of culture medium corresponding to approximately 100 mg of dry cells was harvested by centrifuging at 5000 rpm for 10 min. The cell pellet was washed with distilled water and the cell suspension was centrifuged for another 10 min at 5000 rpm. The cell pellet was resuspended in 4 ml HCl 0.2 N and heated in a water bath set at 85 ºC for 1 h and allowed to cool to room temperature. After centrifuging for 10 min at 5000 rpm and removing supernatant, the cell pellet was resuspended in 2 ml methanol containing 0.2% (w/v) pyrogallol and 1 ml KOH 4 N and transferred to a 14 ml glass vial sealed with a PTFE lined screw cap, heated again for 2 h in a water bath set at 85 ºC for saponification. Following incubation, glass vials were allowed to cool to room temperature. Sterols were then extracted by addition of 5 ml heptane followed by vigorous vortex mixing for 2 min. After 2 h when the heptane layer had clarified, it was transferred to a new glass vial for HPLC analysis.

3.2.9 Analysis of ergosterol

Quantitative determination of ergosterol was carried out by reverse-phase HPLC (Hewlett-Packard HP 1090 chromatograph series II with built in diode array detector and auto-injector). Sterols were separated on a Develosil column (C30-UG-5, Nomura Chemicals, Aichi, Japan) with a mobile phase consisting of methanol and acetonitrile. The analysis was performed at 40 ºC and it was monitored at 280 nm. The amount of ergosterol was determined using absolute calibration curves obtained after each analysis run.

3.2.10 Analysis of sugars and extracellular metabolites

To determine the concentration of sugars and extracellular metabolites in the culture media, 2 ml samples were withdrawn from the fermenter and immediately filtered through a 0.45 µm pore-size cellulose acetate filter (Sartorius AG, Göttingen, Germany). The filtrate was stored at -20 ºC until HPLC analysis. Glucose, galactose, glycerol, pyruvate, succinate, acetate, and ethanol concentrations were determined in a Waters 717 plus Autosampler HPLC system equipped with a Bio-Rad Aminex HPX-87H reverse phase column (Biorad, Hercules, CA) at 60 ºC using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min. Glucose, galactose, glycerol, succinate, and ethanol were detected refractometrically (Waters 410 Differential Refractometer Detector, Millipore Corp., Milford, MA). Pyruvate and acetate were determined spectrophotometrically with a Waters 490E Programmable Multiwavelength Detector set at 210 nm.

3.2.11 Analysis of sesquiterpenes by SPME method

Identification of sesquiterpenes produced by the yeast strains transformed with heterologous sesquiterpene synthases was performed by extracting volatile compounds from the head-space of the samples on 100 µm PDMS fibers (Supelco, Bellefonte, PA, USA). Fibers were conditioned according to the suppliers' instruction. Two ml samples from the culture media were immediately frozen and stored in 4 ml screw cap glass vials at -20 ºC until analysis. The contents of vials were thawed on ice and allowed to equilibrate at room temperature. Extraction was performed for 25 min at 60 ºC while mixing the sample with a small magnet. After extraction, the analytes were thermally desorbed at 250 ºC from SPME fiber into the injector of gas chromatogram in the splitless mode. The oven temperature was held initially at 45 ºC for 1 min, then raised to 130 ºC by a ramp of 10 ºC/min followed by a ramp of 3 ºC/min to 160 ºC. The

oven temperature was further raised to 250 ºC at 10 ºC/min and maintained at this temperature for 5 min to equilibrate.

3.2.12 Analysis of sesquiterpenes in the organic layer

Samples from organic layer were centrifuged for 5 min at 3500 rpm and subsequently analyzed by GC-MS to determine the level of sesquiterpenes during the course of fermentation. GC-MS analyses were run on a Thermo Finnigan Focus GC coupled to a Focus DSQ quadrupole mass spectrometer. Analytes from 1 µl samples were separated on a SLB-5ms capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Supelco, Bellefonte, PA, USA) using helium as carrier gas at the flow rate of 1.2 ml/min. A split/splitless injector was used in the splitless mode. The initial oven temperature was 80 ºC and injector temperature was 250 ºC. After 1 min the oven temperature was increased to 120 ºC at the rate of 10 ºC/min and subsequently increased to 160 ºC at the rate of 3 ºC/min. The oven temperature was finally increased to 270 ºC at the rate of 10 ºC/min and held for 5 min at this temperature. Quantification of compounds was carried out using standard curves generated after each analysis run.

3.2.13 Analysis of methionine

The concentration of methionine in the media was measured by HPLC by the method of Barkholt and Jensen (1989).

3.3 Results

3.3.1 Sesquiterpene synthase genes are functional in the engineered yeast strains

Yeast strains were transformed with individual plasmids bearing one of the three sesquiterpene synthase cDNAs under control of *TPI1* or *GAL1* promoters: a valencene synthase (GFTpsD) and a cubebol synthase (GFTpsC) from grapefruit and a patchoulol synthase (PatTps177) from patchouli. To confirm the functionality of these plasmids the transformed strains were grown in shake flasks containing 100 ml minimal medium and 20 g/L galactose as carbon source. Volatile compounds were adsorbed on SPME fibers and analyzed by GC-MS. All the transformed strains were able to synthesize sesquiterpenes. In the case of valencene synthase, valencene was clearly the major product (Figure 3.1-a) in accordance with the relative high product specificity observed with this enzyme in *in-vitro* assay (data not shown). However, the patchoulol synthase

and the cubebol synthase are multiple product enzymes. The patchoulol synthase produces, in *in-vitro* assays, 14 different sesquiterpenes with patchoulol as major product (Deguerry et al, 2006). Similarly, the cubebol synthase produces mainly cubebol besides at least 14 additional products. Analysis of the volatiles produced by yeast cells transformed with either the patchoulol or cubebol synthase cDNA revealed the same sesquiterpene profile as observed in the *in-vitro* assays. Figures 3.1-b and 3.1-c illustrate the range of sesquiterpene products produced by the cubebol synthase from grapefruit and patchoulol synthase from patchouli, respectively.

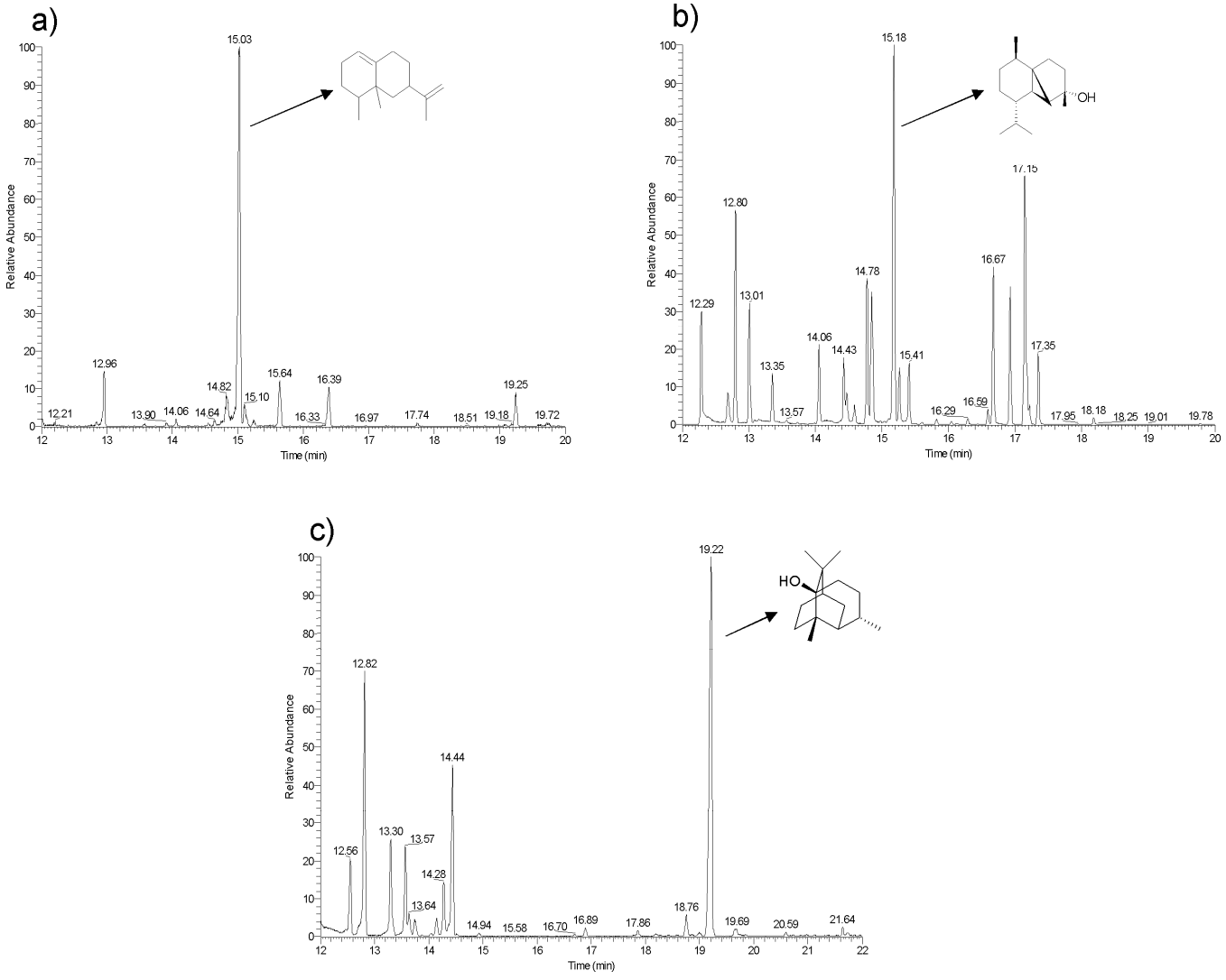

Figure 3.1. GC profile of the sesquiterpenes produced by yeast strains expressing (a) valencene synthase (YIP-0V-02) (b) cubebol synthase (YIP-0C-02) (c) patchoulol synthase (YIP-0P-02)

3.3.2 Minimum methionine concentration for *ERG9* repression

The minimum methionine concentration for adjusting the expression of *ERG9* was determined by growing the *ERG9* repressed yeast cells in shake flasks supplemented with varying amounts of methionine (Figure 3.2).

Repression of *ERG9* led to lower specific growth rates compared to the wild type strain and also reduced the final concentration of biomass in the medium presumably as a result of decreased flux towards ergosterol. Increasing the methionine concentration to 1 mM resulted in an elongation of the lag phase and a lower final biomass concentration. However, there was no further effect on growth profile at concentrations of methionine above 1 mM (Figure 3.2). So the minimum required level of methionine should be 1 mM. However, to maintain the concentration of methionine at the minimum required level for *ERG9* repression during the course of fermentation, 2 mM methionine was chosen for running fermentations.

Repression of *ERG9* resulted in the reduced ergosterol content of yeast strains. For the wild type the ergosterol content was found to be 9.37 ± 0.96 mg ergosterol/g DW, whereas it was found to be 7.48 ± 1.55 mg ergosterol/g DW for YIP-MV-02 when there was no methionine added to the medium and 1.66 ± 0.02 mg ergosterol/g DW when 2 mM methionine was added to the medium.

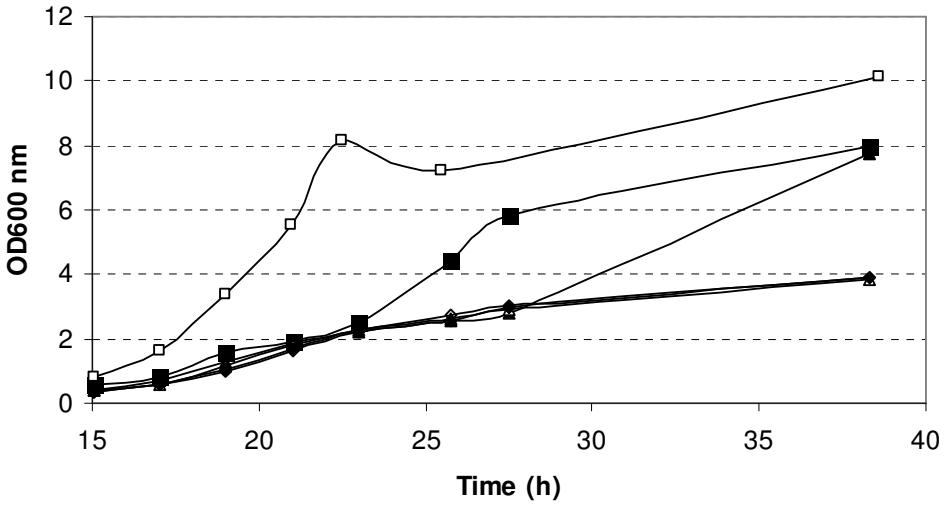

Figure 3.2. Effect of methionine on growth pattern of *ERG9* repressed yeast strain (YIP-MV-02) cultivated in shake flasks containing minimal medium and 20 g/L glucose. (Without methionine: □; 0.25 mM methionine: ■; 0.50 mM methionine: ▲; 1.0 mM methionine: △; 1.5 mM methionine: ◆; 2 mM methionine: ◇)

3.3.3 Characterization of strains expressing synthases on plasmid

Yeast strains transformed with plasmids harboring each of the three sesquiterpene synthase genes under control of either *TPI1* or *GAL1* promoters were characterized in two-phase 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose. Dodecane was added to fermenters as secondary phase. Samples were taken from the organic phase at different times and were analyzed for sesquiterpene content. Figure 3.3 illustrates the profile of sesquiterpene accumulation for the yeast strains expressing sesquiterpene synthases on plasmid.

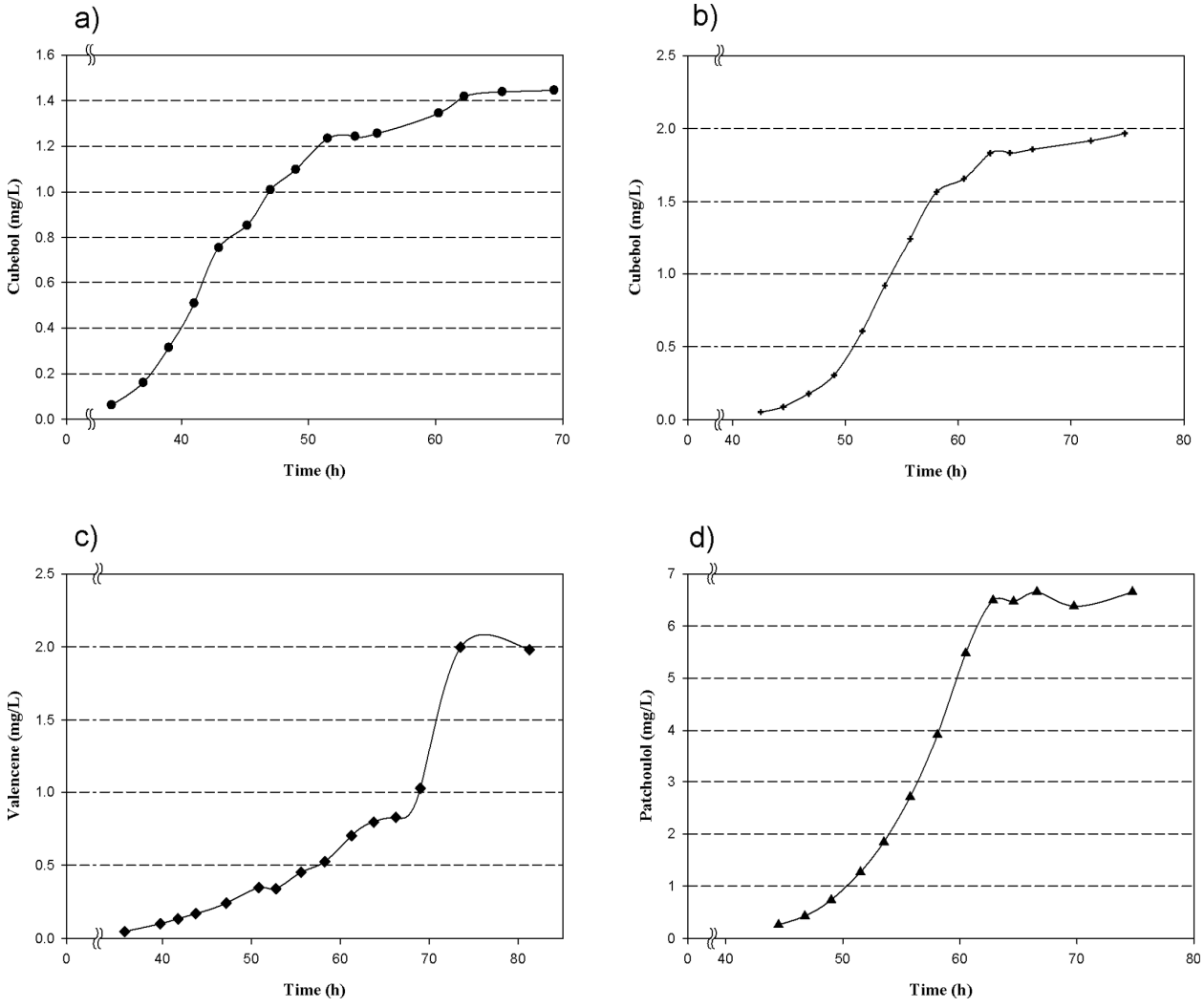

Figure 3.3. Sesquiterpene concentration as a function of time for yeast strains expressing sesquiterpene synthases (a) YIP-0C-01 (b) YIP-0C-02 (c) YIP-0V-02 (d) YIP-0P-02

Table 3.3 summarizes the final concentrations and yields of the sesquiterpenes for the yeast strains harboring synthase genes. However patchoulol constitutes only 37% of the total sesquiterpenes synthesized by the patchoulol synthase (Deguerry et al., 2006) and the total concentration and yields of this sesquiterpene (strain YIP-0P-02) are significantly higher than for the other strains indicating higher activity of patchoulol synthase than valencene and cubebol synthases. In the same way, cubebol represents only 28% of sesquiterpenes produced by the cubebol synthase (unpublished data) and the total quantities of sesquiterpenes produced by strains expressing this enzyme are higher than for strains expressing the valencene synthase indicating that cubebol synthase is also more active than valencene synthase. Total sesquiterpene concentrations were estimated based on the known percentage of the target sesquiterpenes produced by the different sesquiterpene synthases (28% and 37% for cubebol and patchoulol, respectively).

Replacement of *GAL1* promoter with *TPI1* promoter for controlling the expression of cubebol synthase gene reduced the efficiency of sesquiterpene production both in terms of final concentration and yield when galactose was the carbon source (Table 3.3; YIP-0C-01 vs. YIP-0C-02).

Table 3.3. Final concentrations of target sesquiterpenes and total sesquiterpenes and yields of target sesquiterpenes (mg sesquiterpene per gram dry weight or gram consumed galactose in the galactose consumption phase) for yeast strains expressing sesquiterpene synthases.
Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose as carbon source.

Yeast strain	Promoter	Valencene (mg/L)	Cubebol (mg/L)	Patchoulol (mg/L)	Total sesquiterpenes (mg/L)	Y_{XP} (mg/g DW)	Y_{SP} (mg/g galactose)
YIP-0V-02	*GAL1*	2.0			2.0	0.33	0.08
YIP-0C-02	*GAL1*		2.0		7.1	0.33	0.07
YIP-0C-01	*TPI1*		1.5		5.4	0.21	0.04
YIP-0P-02	*GAL1*			6.7	18.1	1.21	0.31

3.3.4 *ERG9* repression diverts FPP towards farnesol

Analysis of dodecane layer from two-phase batch fermentation of the *ERG9* repressed strain without any sesquiterpene synthase (YIP-M0-04) revealed formation of farnesol as an FPP-derived compound during fermentation.

The yield of farnesol when cells were assimilating galactose as carbon source was 10.90 mg/g DW and the final titer was 22.6 mg/L. The concentration and yield of the compounds directly derived from FPP are higher when *ERG9* is repressed. Taken together with the reduced ergosterol content of the *ERG9* repressed strains, this confirms the possibility of using this strategy for enhancing FPP availability for the sesquiterpene synthases.

3.3.5 Combination of *ERG9* repression and sesquiterpene synthase expression

ERG9 repression was shown to result in formation of FPP derived by-products and therefore it is likely that FPP availability for sesquiterpene synthases was improved. Hence, combination of *ERG9* repression and sesquiterpene synthase expression was expected to give rise to higher accumulation of sesquiterpenes. To investigate this hypothesis the YIP-M0-04 strain was transformed with plasmids each bearing one of the three sesquiterpene synthase genes and the constructed strains were characterized in 5 L batch fermenters. None of the sesquiterpene synthase genes were able to convert all the available FPP to the target sesquiterpenes and again farnesol was observed as FPP derived by-product (Figure 3.4).

Comparing the final concentrations of desired sesquiterpenes and farnesol produced by *ERG9* repressed strains and the corresponding strains where *ERG9* is under control of the endogenous promoter (Table 3.3 and Table 3.4) indicate that except for the patchoulol synthase, repression of *ERG9* did not lead to significantly higher amounts of sesquiterpenes but mainly resulted in the diverting of FPP to farnesol. This again shows that patchoulol synthase activity in the yeast cells is higher than the activities of the other two sesquiterpene synthases.

Replacing the *GAL1* promoter in the YIP-MV-01 strain with *TPI1* promoter (YIP-MV-02) resulted in lower amount of valencene and higher yield and concentration of farnesol.

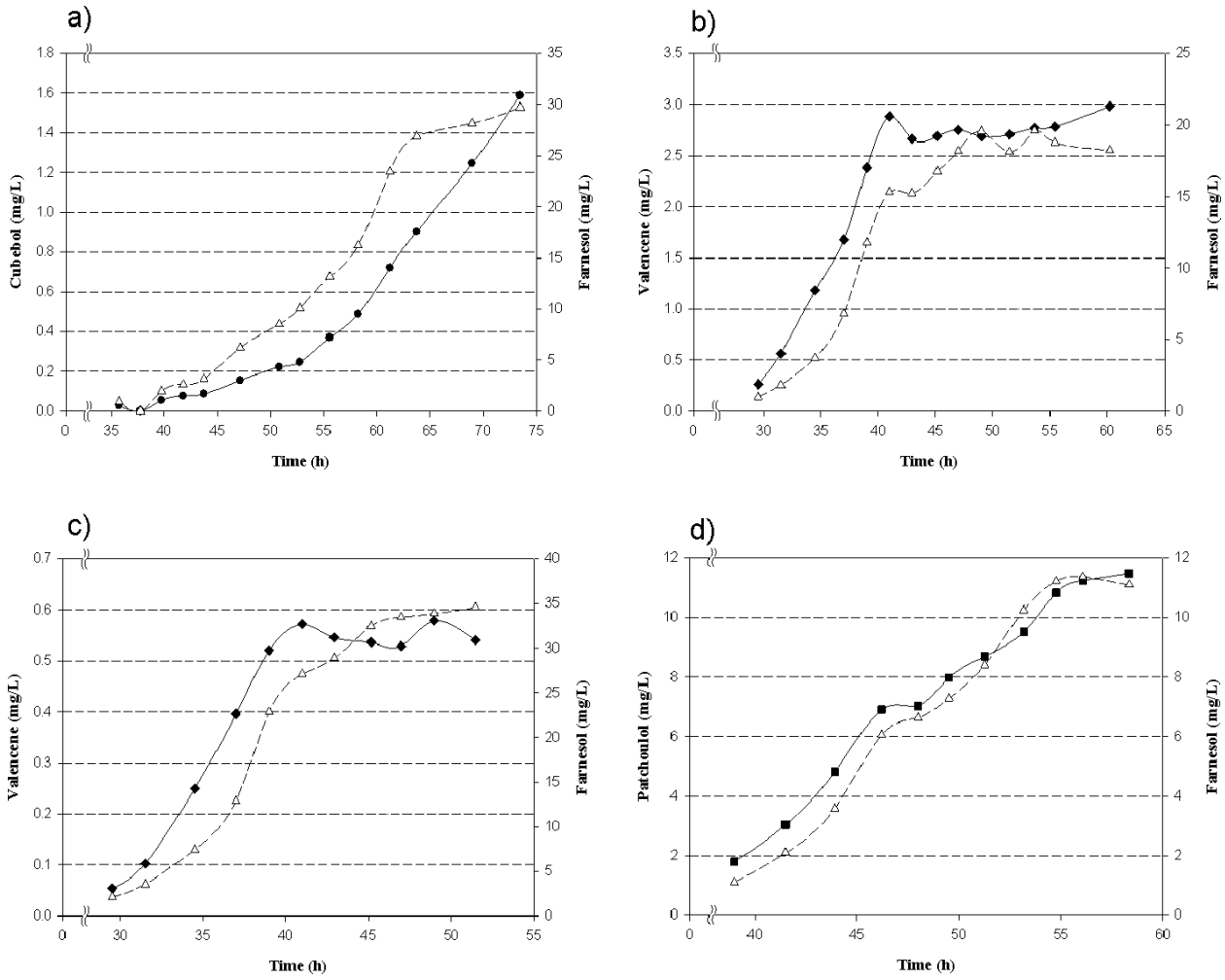

Figure 3.4. Target sesquiterpenes and farnesol concentrations as a function of time for *ERG9* repressed yeast strains expressing sesquiterpene synthases (a) YIP-MC-02 (b) YIP-MV-01 (c) YIP-MV-02 (d) YIP-MP-01; Valencene (♦) Cubebol (●) Patchoulol (■) Farnesol (△)

Table 3.4. Final concentrations and yields of sesquiterpenes for the *ERG9* repressed yeast strains. Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose.

Yeast strain	Sesquiterpene synthase	Promoter	Final titer of target sesquiterpene (mg/L)	Total sesquiterpenes (excluding farnesol) (mg/L)	Farnesol (mg/L)	Yield of target sesquiterpene on biomass (mg/g DW)	Yield of target sesquiterpene on galactose (mg/g galactose)	Yield of farnesol on biomass (mg/g DW)	Yield of farnesol on galactose (mg/g galactose)
YIP-MC-02	Cubebol	*TPI1*	1.6	5.7	29.9	0.39	0.23	13.15	5.30
YIP-MV-01	Valencene	*GAL1*	3.0	3.0	19.6	3.13	0.67	9.05	3.77
YIP-MV-02	Valencene	*TPI1*	0.6	0.6	34.6	0.38	0.14	14.93	5.99
YIP-MP-01	Patchoulol	*GAL1*	11.5	31.1	11.1	6.25	2.13	4.98	2.06
YIP-M0-04	----	----	----	0	22.6	0	0	10.90	1.76

3.3.6 Regulating methionine concentration alters the flux towards sesquiterpenes

ERG9 down-regulation was not concomitant with substantial improvement in the final concentrations of the target sesquiterpenes, but comparing the yields (Table 3.3 and Table 3.4) demonstrates that there was some increase in the yields. Plotting the sesquiterpene concentration versus dry weight of biomass or galactose concentration allowed calculation of yields and this revealed two different linear phases during the galactose consumption phase (Figure 3.5) suggesting that the rate of sesquiterpene accumulation in the organic phase dropped at one time during the fermentation. It was speculated that methionine was metabolized by the cells and the subsequent reduced levels of methionine would relieve a true repression of the *ERG9* gene and consequently lowered the accumulation rate of sesquiterpenes. Analyzing the methionine concentration in the culture media confirmed that there was a total consumption of methionine (data not shown). To examine the effect of methionine concentration on the level of products we therefore ran fermentations with the YIP-MP-01 strain using higher amounts of methionine added to the medium at different times during fermentation. Maintaining the methionine concentration at higher level during the fermentation resulted in a 47% increase in the final patchoulol titer and a 82% increase in the final farnesol titer. Thus, the final concentrations of patchoulol and farnesol reached to 16.9 and 20.2 mg/L, respectively.

3.3.7 Farnesol is converted to other by-products at the end of fermentation

It was noticed that there is a drastic drop in the farnesol concentration for the *ERG9* repressed strains when all carbon sources had been depleted. This decrease could not be a result of evaporation of farnesol from the organic phase since the same pattern was not observed for other compounds. We speculated that at the end of fermentation and after depletion of all carbon sources, farnesol is further converted to other related compounds and appearance of a second peak during GC-MS analysis for FPP-derived compounds at the end of the fermentations supported this hypothesis (Figure 3.6).

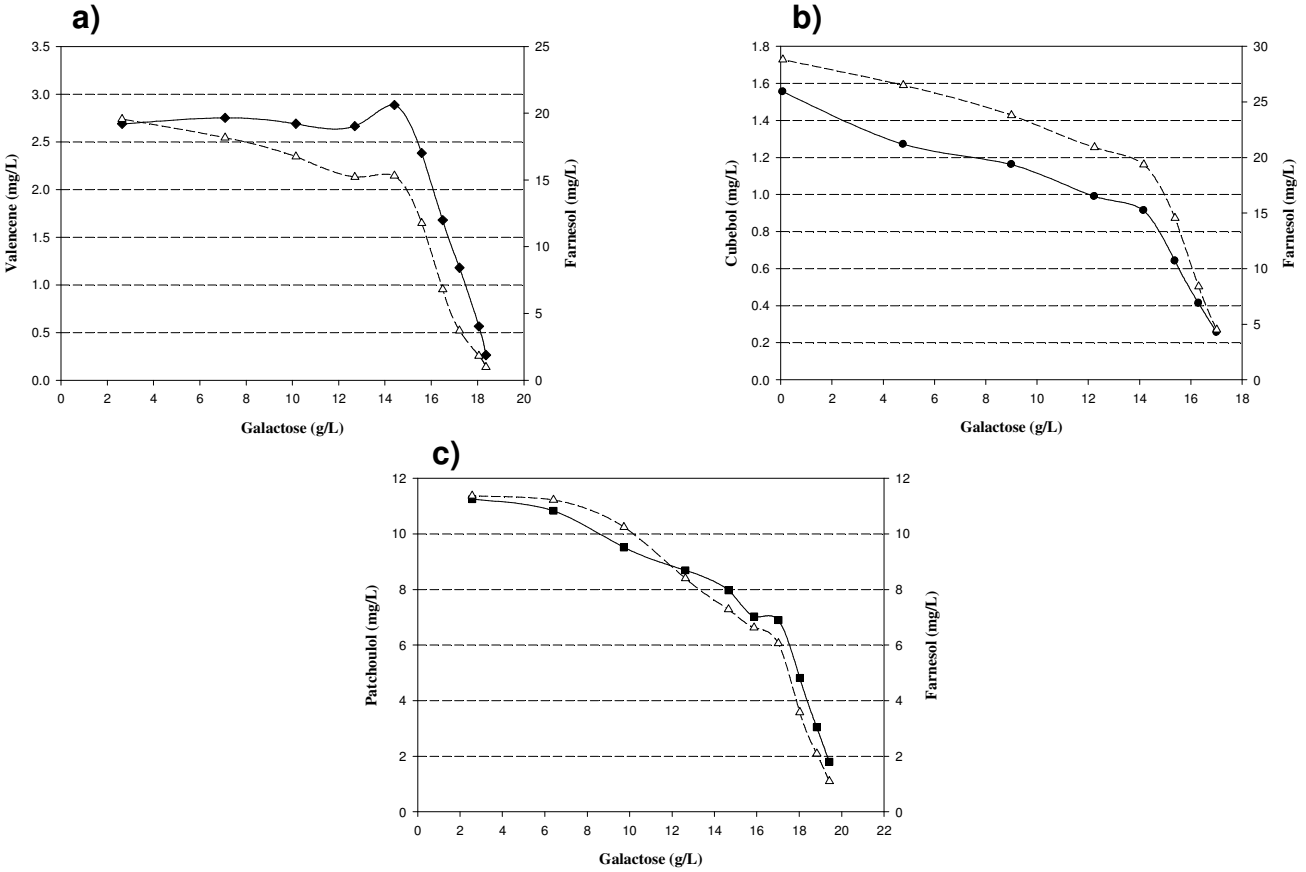

Figure 3.5. Target sesquiterpenes and farnesol concentrations as a function of galactose concentration for calculating yields on galactose. (a) YIP-MV-01 (b) YIP-MC-02 (c) YIP-MP-01; Valencene (♦)Cubebol (●) Patchoulol (■) Farnesol (△)

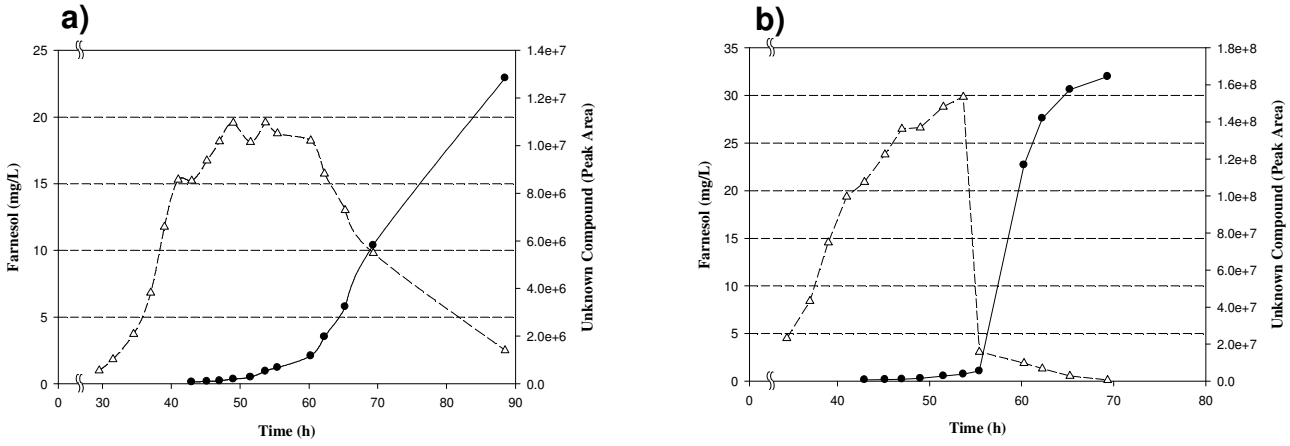

Figure 3.6. Farnesol degradation at the end of fermentation and appearance of an unknown compound. (a) YIP-MV-01 (b) YIP-MC-02; Farnesol (△) Unknown compound (●)

3.3.8 Galactose is preferred carbon source for biosynthesis of sesquiterpenes

Galactose is an expensive carbon source and *S. cerevisiae* exhibits a lower specific growth rate on this carbon source. Therefore for industrial fermentation it would be desirable to use glucose as carbon source. We investigated the possibility of using glucose in the media by growing yeast cells expressing cubebol and valencene synthases under control of the *TPI1* promoter using glucose rather than galactose as carbon source. However, the results showed that both the final titer and the yield were lower when cells were grown on glucose (Table 3.4 and Table 3.5).

Table 3.5. Final concentrations and yields of sesquiterpenes and farnesol for the *ERG9* repressed yeast strains expressing sesquiterpene synthases under control of *TPI1* promoter. Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L glucose.

Yeast strain	Valencene (mg/L)	Cubebol (mg/L)	Farnesol (mg/L)	Yield of corresponding sesquiterpene on glucose (mg/g glucose)	Yield of farnesol on glucose (mg/g glucose)
YIP-0C-01		0.7		0.03	
YIP-MC-02		1.5	20.0	0.08	0.82
YIP-MV-02	0.2		15.7	0.01	0.84

3.4 Discussion

In this study we report heterologous production of three different plant sesquiterpenes in the yeast *S. cerevisiae*. The corresponding sesquiterpene genes for cubebol, valencene and patchoulol were expressed in yeast and were shown to be functional, and this is the first instance of a cubebol synthase being expressed for heterologous production of cubebol.

Patchoulol and cubebol synthases produced several products besides their main products, i.e. patchoulol and cubebol, respectively. The ability of sesquiterpene synthases to make multiple products has been well documented and is probably a consequence of premature quenching of the highly unstable carbocationic intermediates that are generated from FPP (Davis and Croteau, 2000)**.** This could also represent adaptation of these enzymes through evolutionary mechanisms to generate the maximum number of products using the minimum genetic and enzymatic machinery (Steele et al., 1998). Indeed, ability of certain enzymes to catalyze secondary reactions besides their primary reactions on a single active site is not a rare phenomenon and has

been termed catalytic promiscuity (Copley, 2003) and multiple product formation by sesquiterpene synthases could also be an example for this feature.

The availability of FPP which is the precursor for all sesquiterpene synthases was enhanced by replacement of the native *ERG9* promoter with a regulatable *MET3* promoter and repressing the promoter with the presence of 2 mM methionine. The ergosterol content was drastically reduced as a consequence of this repression. Nevertheless, this strategy did not improve appreciably the production of the target sesquiterpenes but resulted in further production of farnesol as an FPP-derived by-product. Consistent with this finding are accumulation of farnesol in *Candida albicans* (Buurman et al., 2004; Hornby et al., 2003) as a consequence of treatment with zaragozic acid, which is potent inhibitor of squalene synthase. Furthermore, deletion of *ERG9* gene in *S. cerevisiae* was reported to lead to accumulation of farnesol in *S. cerevisiae* (Song, 2003). Poor expression of the plant sesquiterpene genes and also low catalytic activity of sesquiterpene synthases may explain why sesquiterpene synthesis was not improved significantly after repression of *ERG9*. The use of codon optimized sesquiterpene synthases may help in diverting more FPP towards the desired sesquiterpenes. The production of amorphadiene in yeast was significantly enhanced when the codon usage of the amorphadiene synthase was optimized (Martin et al., 2001; Martin et al., 2003).

Metabolism of methionine during the growth phase relieved the repression of *ERG9*, which was accompanied by a reduction in the rate of sesquiterpene synthesis. Further experiments showed that it was possible to improve the target sesquiterpene production by supplying more methionine during fermentation. Under these conditions the final titer of patchoulol and farnesol reached to 16.9 and 20.2 mg/L, respectively. More precise regulation of methionine should improve further the efficiency of sesquiterpene biosynthesis.

The effect of replacing galactose as an expensive carbon source with glucose, which is less expensive and is faster metabolized by cells, on the sesquiterpene biosynthesis was investigated. However, higher final titer and yield were observed when cells were grown on galactose.

Considering that the reported titers and yields have been achieved only by down-regulating one gene in the pathway, this work demonstrates the capacity of yeast as a cell factory for the production of sesquiterpenes.

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Chapter 4

Overexpression of *tHMG1* enhances heterologous production of sesquiterpenes in *S. cerevisiae*

4.1 Introduction

The yeast *S. cerevisiae* has been used in several studies for heterologous production of sesquiterpenes (Jackson et al., 2003; Ro et al., 2006; Lindahl et al., 2006; Takahashi et al., 2007; Shiba et al., 2007; Asadollahi et al., 2008). Although expression of heterologous sesquiterpene synthase genes in yeast allows production of the corresponding sesquiterpenes, the titer is not sufficient to support a viable industrial process for the production of sesquiterpenes by yeast. It is therefore necessary to increase the supply of the precursor for the production of sesquiterpenes. Sesquiterpenes are derived from FPP, which is an intermediate of the sterol pathway. The sterol biosynthetic pathway in yeast is initiated with the condensation of two molecules of acetyl-CoA and ends with ergosterol as the end product of the pathway after more than 20 steps (Daum et al., 1998). This pathway is divided into two distinct portions. The first part of the pathway, which is also called the mevalonate pathway, leads to the production of FPP but is also responsible for providing intermediates required for the biosynthesis of essential constituents of cell such as heme A, ubiquinone, dolichols, farnesylated and geranylgeranylated proteins (Maury et al., 2005; Kennedy et al., 1999). Regulation of the mevalonate pathway in eukaryotes is very complex, but

HMG-CoA reductase and FPP branch-point represent two key regulation sites in the pathway (Maury et al., 2005).

HMG-CoA reductase, which catalyzes the conversion of HMG-CoA to mevalonate, is a highly regulated enzyme that is generally considered to represent the main flux controlling step in the pathway. *S. cerevisiae* possesses two isozymes of HMG-CoA reductase, Hmg1p and Hmg2p, encoded by the *HMG1* and *HMG2* genes, respectively. At aerobic conditions Hmg1p has the highest activity (Hampton et al., 1996). Both the yeast isozymes have a C-terminal catalytic domain and an N-terminal domain which consists of several transmembrane spans to anchor the protein in the endoplasmic reticulum (Hampton and Rine, 1994). Several studies have shown that overexpression of the region of gene encoding the catalytic domain of Hmg1p leads to enhanced isoprenoid production in yeast (Jackson et al., 2003; Ro et al., 2006; Verwaal et al., 2007).

The FPP branch-point represents another regulatory site in the mevalonate pathway where several enzymes compete for FPP as substrate. However, most of the FPP is required for the sterol biosynthesis and hence minimizing the flux towards sterols would provide more FPP for the other enzymes of the branch-point including sesquiterpene synthases.

We have successfully reported production of three plant sesquiterpenes namely valencene, cubebol and patchoulol in yeast by expression of the corresponding sesquiterpene synthase genes. Minimizing the carbon flux towards sterols through attenuated expression of *ERG9*, which encodes squalene synthase, stimulated sesquiterpene production in yeast. This was done by replacing the endogenous *ERG9* promoter with a regulatable *MET3* promoter that is repressed in the presence of methionine (Asadollahi et al., 2008). In this study, we further studied cubebol production in yeast. The mevalonate pathway in the cubebol producing yeast strain was deregulated by overexpression of the truncated *HMG1* (*tHMG1*) both from the genome and using plasmid based expression under control of strong promoters. The overexpression of *tHMG1* was combined with the down-regulation of *ERG9* in order to further improve cubebol production. The constructed yeast strains were characterized in batch fermenters and the effect of the modifications were studied on cubebol production as well as accumulation of ergosterol and squalene.

4.2 Materials and methods

4.2.1 Plasmid construction

The plasmid pIP025 was constructed by cloning GFTpsC in a pESC-URA vector (Stratagene) under control of the *GAL1* promoter using *BamH*I and *Xho*I restriction sites. Overexpression of the catalytic domain of HMG-CoA reductase (*tHMG1*) was performed using gap repair technique taking the advantage of high efficiency homologous recombination in yeast. The *tHMG1* gene was amplified from the genomic DNA of *S. cerevisiae* CEN.PK113-7D using the primers 5'tctggcgaagaattgttaattaagagctcaTTAGGATTTAATGCAGGTGACGGAC3' and 5'cactaaagggcggccgcactagtatcgatgACT***ATG***GACCAATTGGTGAAAACTG3'. The lower case letters in the primers show the homologous overhang used for gap repair. The truncated coding region has an engineered Met start codon and then continues with the natural coding region from codon 531 (Asp) to the natural stop codon. The PCR conditions were in accordance with the Phusion™ Hot Start High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland). pESC-URA plasmid was digested with *Cla*I and *Sac*I restriction enzymes. The PCR product and the digested expression vector were separated by gel electrophoresis and gel purified using the QIAEX® II Gel extraction kit (Qiagen, Hilden, Germany). Homologous recombination of PCR product into the digested expression vector after transformation of CEN.PK113-5D with both fragments resulted in pIP026 plasmid.

The plasmid pIP028 was obtained by cloning GFTpsC in pIP026 plasmid under control of the *GAL1* promoter using *BamH*I and *Xho*I restriction sites. The plasmids pIP031 and pIP032 have been described previously (Asadollahi et al., 2008).

Sequences of the genes GFTpsD (valencene synthase) and GFTpsC (cubebol synthase) can be obtained from GenBank: accession numbers CQ813508 and CQ813505, respectively.

4.2.2 Integration of *tHMG1* into genome

For genomic integration of *tHMG1* under control of *TPI1* promoter a plasmid bearing a truncated version of *HMG1*, associated with a selective marker, *Kl URA3*, was constructed. *tHMG1* was PCR amplified from CEN.PK 113-7D genomic DNA using primers containing overhangs with *EcoR*I restriction site on one side (5'accggaattcACT***ATG***GACCAATTGGTGAAAACTG3') and *Nhe*I restriction site on the other side (5'tctagcttagcCACATGGTGCTGTTGTGCTT3'). After digestion with *EcoR*I and *Nhe*I, the PCR fragments were ligated into pYX212 plasmid, in

between the two restriction sites *Eco*RI and *Nhe*I. *Kl URA3* was further cloned into pYX212 bearing *tHMG1* at the *Nhe*I restriction site. *Kl URA3* flanked by two direct repeats was first PCR amplified from the plasmid pWJ1042 (Reid et al., 2002) using the couple of primers 5'tctagctagcTTCGGCTTCATGGCAATTCCCG3' and 5'tctagctagcTAACGCCAGGGTTTTCCCAGTCAC3'. After digestion with *Nhe*I, *Kl URA3* flanked by the two direct repeats was ligated into the *Nhe*I restriction site of pYX212 bearing *tHMG1*. This plasmid was named pTTU.

In order to integrate the *TPIp-tHMG1-Kl URA3* construct into the *URA3* locus, fusion PCR and the bipartite gene targeting method (Erdeniz et al., 1997) was used. *TPIp-tHMG1-Kl URA3* was amplified from the plasmid pTTU in two separate, but overlapping, fragments (Figure 4.1) using the primers listed in Table 4.1. Furthermore, 500 bp upstream and downstream of *URA3* on the genome of *S. cerevisiae* were amplified. The four resulting PCR fragments were gel-purified and fused together in pairs by fusion PCR (Figure 4.1). The primers URA3sl500 and 3'int KL URA3 were used for fusion of the "left fragment"; primers 5'int KL URA3 and URA3rr500 were used for fusion of the "right fragment". The resulting two fusion PCR fragments were purified after gel electrophoresis. *S. cerevisiae* strain CEN.PK 113-5D was subsequently transformed with the fusion PCR fragments. Transformants were selected on SC-ura medium after incubation at 30 °C for 2-4 days. Several transformants were selected and their genomic DNA was isolated. Correct integrants were identified by PCR using both external primers (URA3sl500 and URA3rr500) and a combination of external and internal primers (5'int KL URA3 and URA3rr500).

Subsequently, correct transformants were restreaked on a medium containing 5-FOA for the loop out of the *Kl URA3* marker and the reuse of uracil auxotrophy.

4.2.3 Strain construction

The native promoter of *ERG9* gene was replaced with a regulatable *MET3* promoter and thus the strain YIP-M0-04 was obtained (Asadollahi et al., 2008).

Strains YIP-MV-07 and YIP-MC-13 were obtained by transforming YIP-M0-03 with the pIP031 and pIP032 plasmids, respectively. Strains YIP-0C-04, YIP-MC-05, YIP-MC-07, YIP-MC-08, YIP-MC-09, and YIP-MC-10 were constructed by transforming their corresponding backgrounds with either pIP025 or pIP028 plasmids. Table 4.2 lists the strains used in this study.

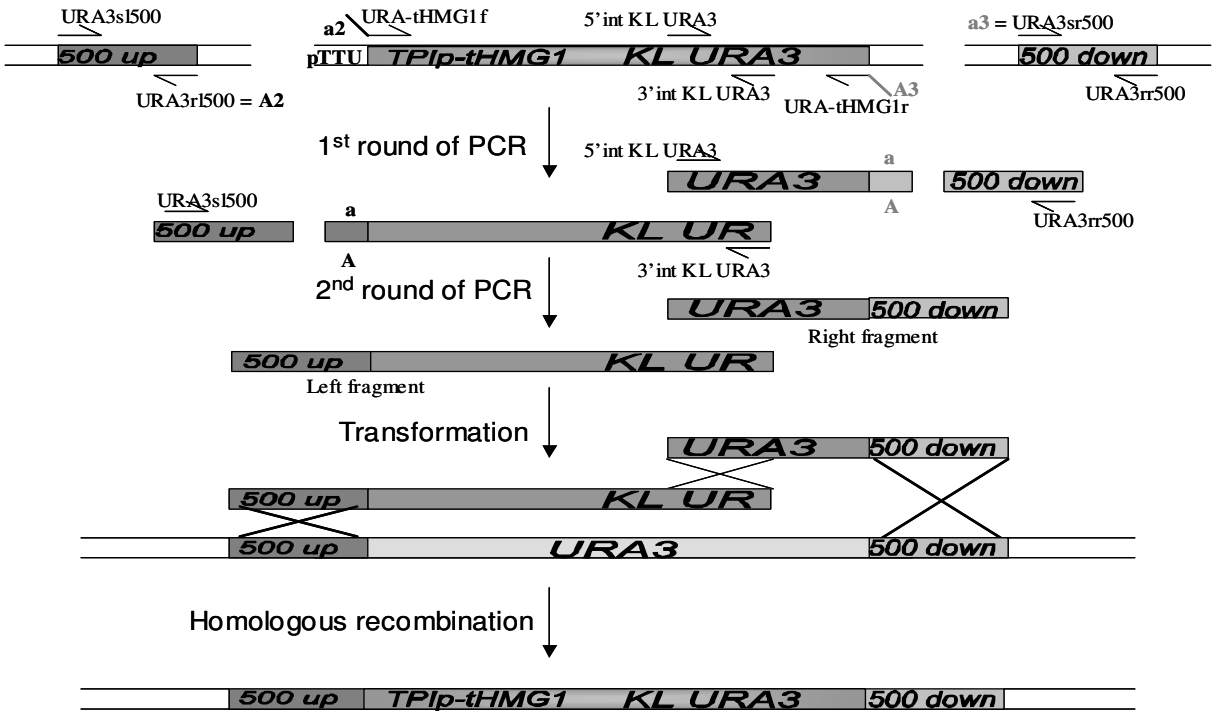

Figure 4.1. Insertion of *TPIp-tHMG1-Kl URA3* into the *URA3* locus of *S. cerevisiae*. The *TPIp-tHMG1-KL URA3* construct is amplified from pTTU in two separate, but overlapping, reactions using primers at the extremities with adaptamers homologous to the insertion site. 500 bp up- and downstream from the insertion site are amplified as well. After a second round of PCR where the four resulting fragments are fused together in pairs the two fusion fragments are transformed into *S. cerevisiae*.

Table 4.1. Primers for amplification of fusion PCR fragments.

Primer	Sequence
URA-tHMG1s	atgtcgaaagctacatataaggaacgtgctgctactcatcctagtcctgtGAAAAGTGCCACCTGACGTC
URA-tHMG1r	ttgctggccgcatcttctcaaatatgcttcccagcctgcttttctgtaacgTGTAAAACGACGGCCAGTGAG
5'int KL URA3	CTTGACGTTCGTTCGACTGATGAGC
3'int KL URA3	GAGCAATGAACCCAATAACGAAATC
URA3sl500	AAACGACGTTGAAATTGAGGCTACTGCG
URA3rl500	GGACTAGGATGAGTAGCAGCACGTTCC
URA3sr500	GGGAAGCATATTTGAGAAGATGCGGC
URA3rr500	GGAAACGCTGCCCTACACGTTCGC

The sequence of the primer used for amplification of the DNA fragments is given in capital letters, while the overhangs used in fusion PCR are in small letters.

Table 4.2. Strains used in this study

Strain	Genotype	Plasmid	Plasmid description	Reference
YIP-00-03 (CEN.PK113-5D)	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	none		Peter Kötter[a]
YIP-M0-04	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	none		Asadollahi et al., 2008
YIP-0V-01	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP031	pYX212 2µ *URA3* P_{TPI1}-GFTpsD	Asadollahi et al., 2008
YIP-0C-01	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP032	pYX212 2µ *URA3* P_{TPI1}-GFTpsC	Asadollahi et al., 2008
YIP-M0-03	*MAT***a** *MAL2-8*^{c} *SUC2, ura3 ::*P_{TPI1}*-tHMG1*	none		This study
YIP-MV-07	*MAT***a** *MAL2-8*^{c} *SUC2, ura3 ::*P_{TPI1}*-tHMG1*	pIP031	pYX212 2µ *URA3* P_{TPI1}-GFTpsD	This study
YIP-MC-13	*MAT***a** *MAL2-8*^{c} *SUC2, ura3 ::*P_{TPI1}*-tHMG1*	pIP032	pYX212 2µ *URA3* P_{TPI1}-GFTpsC	This study
YIP-0C-04	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP025	pESC-URA 2µ *URA3* P_{GAL1}- GFTpsC	This study
YIP-MC-05	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP028	pESC-URA 2µ *URA3* P_{GAL1}-GFTpsC P_{GAL10}*-tHMG1*	This study
YIP-MC-07	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	pIP025	pESC-URA 2µ *URA3* P_{GAL1}- GFTpsC	This study
YIP-MC-08	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	pIP028	pESC-URA 2µ *URA3* P_{GAL1}-GFTpsC P_{GAL10}*-tHMG1*	This study
YIP-MC-09	*MAT***a** *MAL2-8*^{c} *SUC2, ura3 ::*P_{TPI1}*-tHMG1*	pIP025	pESC-URA 2µ *URA3* P_{GAL1}- GFTpsC	This study
YIP-MC-10	*MAT***a** *MAL2-8*^{c} *SUC2, ura3 ::*P_{TPI1}*-tHMG1*	pIP028	pESC-URA 2µ *URA3* P_{GAL1}-GFTpsC P_{GAL10}*-tHMG1*	This study

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4.2.4 Media for Shake flasks

Baffled, cotton-stopped, 500 ml Erlenmeyer flasks were used for preparing precultures and also for preparing samples for ergosterol measurement and HMG-CoA reductase assays. The shake flasks were containing 100 ml medium with the following compositions: 7.5 g/L $(NH_4)_2SO_4$; 14.4 g/L KH_2PO_4; 0.5 g/L $MgSO_4.7H_2O$; 2 ml/L trace metal solution; 1 ml/L vitamin solution and 50 µl/L synperonic antifoam (Sigma, St. Louis, MO). The pH of the mineral medium was adjusted to 6.50 by adding 2M NaOH and autoclaved separately from carbon source solution. Vitamin solution was filter sterilized and aseptically added to the medium after autoclavation. Shake flasks were incubated in a shaking incubator at 30 ºC and 150 rpm.

4.2.5 Media for batch cultivations

A defined minimal medium as described by Verduyn et al. (1992) containing 20 g/L of galactose as the sole carbon source was used for all batch fermentations. The media had the following compositions: 5 g/L $(NH_4)_2SO_4$; 3 g/L KH_2PO_4; 0.5 g/L $MgSO_4.7H_2O$; 1 ml/L trace metal solution; 1 ml/L vitamin solution and 50 µl/L synperonic antifoam. Expression of *ERG9* was repressed by supplementing media with 2 mM filter sterilized methionine.

Galactose was autoclaved separately from the medium and subsequently added to the fermenter, as was the case for the vitamin solution that was added after filter sterilization.

4.2.6 Batch fermentations

Batch fermentations were carried out in well-controlled 5 L in-house manufactured glass bioreactors with a working volume of 4 L. The bioreactors were equipped with two disk-turbine impellers and 4 baffles to ensure proper mixing. The pH was controlled between 4.95 and 5.05 by automatic addition of 2M NaOH. The temperature was kept constant at 30 ºC. The air flow was 4 L/min (1 vvm) and was sterilized by filtration and the off gas passed through a condenser. Carbon dioxide and oxygen concentrations in the off-gas were determined by a Brüel & Kjær acoustic gas analyzer (Brüel & Kjær, Nærum, Denmark). Batch fermenters were inoculated to an initial OD_{600} of 0.02 from a liquid preculture. Three hundred milliliters of dodecane was added aseptically to the media at OD_{600} 0f 1 ± 0.1.

4.2.7 OD and dry weight determinations

The OD of samples was determined at 600 nm in duplicate by using a Hitachi U-1100 spectrophotometer. Dry weight measurement was achieved by using 0.45 µm pore-size nitrocellulose filters (Sartorius AG, Göttingen, Germany) according to the method described by Dynesen et al. (1998).

4.2.8 Analysis of galactose and ethanol

To determine the concentration of sugars and extracellular metabolites in the culture media, 2 ml samples were withdrawn from the fermenter and immediately filtered through a 0.45 µm pore-size cellulose acetate filter (Sartorius AG, Göttingen, Germany). The filtrate was stored at -20 ºC until HPLC analysis. Analysis was performed in a Waters 717 plus Autosampler HPLC system equipped with a Bio-Rad Aminex HPX-87H reverse phase column (Biorad, Hercules, CA) at 60 ºC using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min. Galactose and ethanol were detected refractometrically (Waters 410 Differential Refractometer Detector, Millipore Corp., Milford, MA).

4.2.9 Analysis of sesquiterpenes in the organic layer

Samples from organic layer were centrifuged for 5 min at 3500 rpm and subsequently analyzed by GC-MS to determine the level of sesquiterpenes during the course of fermentation. GC-MS analyses were run on a Thermo Finnigan Focus GC coupled to a Focus DSQ quadrupole mass spectrometer. Analytes from 1 µl samples were separated on a SLB-5ms capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Supelco, Bellefonte, PA, USA) using helium as carrier gas at the flow rate of 1.2 ml/min. A split/splitless injector was used in the splitless mode. The initial oven temperature was 80 ºC and injector temperature was 250 ºC. After 1 min the oven temperature was increased to 120 ºC at the rate of 10 ºC/min and subsequently increased to 160 ºC at the rate of 3 ºC/min. The oven temperature was finally increased to 270 ºC at the rate of 10 ºC/min and held for 5 min at this temperature.

Quantification of compounds was carried out using standard curves generated after each analysis run.

4.2.10 Extraction of sterols

Baffled, cotton-stopped, shake flasks containing 100 ml minimal medium and 20 g/L of glucose or galactose as carbon source were inoculated with overnight precultures to an initial OD_{600} of 0.05 and incubated for 24 h in a shaking incubator at 30 ºC and 150 rpm. A certain volume of culture media corresponding to approximately 100 mg of dry cells was harvested by centrifuging at 5000 rpm for 10 min. The cell pellet was washed with distilled water and the cell suspension was centrifuged for another 10 min at 5000 rpm. The cell pellet was resuspended in 4 ml HCl 0.2 N and heated in a water bath set at 85 ºC for 1 h and then allowed to cool to room temperature. After centrifuging for 10 min at 5000 rpm and removing supernatant, the cell pellet was resuspended in 2 ml methanol containing 0.2% (w/v) pyrogallol and 1 ml KOH 4 N and transferred to a 14 ml glass vial sealed with a PTFE lined screw cap, heated again for 2 h in a water bath set at 85 ºC for saponification. Following incubation, glass vials were allowed to cool to room temperature. Sterols were then extracted by addition of 5 ml heptane followed by vigorous vortex mixing for 2 min. After 2 h when the heptane layer had clarified, it was transferred to a new glass vial for HPLC and GC-MS analyses.

4.2.11 Analysis of ergosterol

Quantitative determination of ergosterol was carried out by reverse-phase HPLC (Hewlett-Packard HP 1090 chromatograph series II with built in diode array detector and auto-injector). Sterols were separated on a Develosil column (C30-UG-5, Nomura Chemicals, Aichi, Japan) with a mobile phase consisting of methanol and acetonitrile. The analysis was performed at 40 ºC and it was monitored at 280 nm. The amount of ergosterol was determined using absolute calibration curves obtained after each analysis run.

4.2.12 Analysis of squalene

Samples from sterol extraction were injected into a Thermo Finnigan Focus GC coupled to a Focus DSQ quadrupole mass spectrometer for squalene analysis and determination. Sterols were separated on a SLB-5ms capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Supelco, Bellefonte, PA, USA) using helium as carrier gas at the flow rate of 1.2 ml/min. A split/splitless injector was used in the splitless mode. The initial oven temperature was 80 ºC and injector temperature was 250 ºC. After 1 min the oven temperature was increased to 270 ºC

at the rate of 10 ºC/min. The oven temperature was then held for 20 min at this temperature to equilibrate.

4.2.13 HMG-CoA reductase activity

Baffled, cotton-stopped, shake flasks containing 100 ml minimal medium and 20 g/L of glucose as carbon source were inoculated with overnight precultures to an initial OD_{600} of 0.02 and incubated for 24 h in a shaking incubator at 30 ºC and 150 rpm. Fifty milliliters of culture medium was harvested by centrifuging at 5000 rpm for 10 min. The cell pellets were washed twice with 25 ml of 50 mM phosphate buffer (pH 7.0). After removing the supernatant, cells were resuspended in 300 µl of lysis buffer (100 mM phosphate buffer, pH 7.0; 1 mM Na_2EDTA, pH 8.0; 5mM DTT). The suspension was then transferred to a 2 ml FastPrep® tube and precooled glass beads (0.25-0.50 mm) were added to reach the meniscus. The cells were broken by vortexing in FastPrep® instrument (FP 120, BIO 101, Savant, Holbrook, NY) at the speed set to 5.0 m/sec for 3×20 sec separated by periods of cooling on ice. An additional 300 µl of lysis buffer was added to assist the recovery of the extract. The extract was transferred to a new Eppendorf tube and Nonidet P-40 was added to 0.5% (w/v) and incubated on ice for 1 h. The extract was centrifuged at 10000 g for 2 min at 4 ºC. Activity assays were modified from the procedure of Quain and Haslam (1979). For the assay a reaction buffer containing 100 mM KPO_4 buffer, pH 7.0, 0.5% (w/v) Nonidet P-40, 5 mM DTT was prepared. Stock solutions of NADPH and HMG-CoA in KPO_4 buffer, 3mM, pH 7.0, were prepared in separate tubes. Assays were done in 1 ml (1 cm path length) quartz cuvettes by adding reaction buffer and 150 µM NADPH. The cuvette temperature was kept constant at 30 ºC by circulating water. The cuvette was put in the place at 30 ºC for 10 min to equilibrate. Then different amounts of cell extract (50, 100, and 150 µl) were added to the cuvette. After 5 min of incubation at 30 °C to stabilize the endogenous oxidation of NADPH, 150 µM HMG-CoA was added to the cuvette as substrate. The initial change in absorbance at 340 nm due to oxidation of NADPH was recorded.

The protein content of cell extract was determined using the Lowry method (Lowry et al., 1951) using BSA as standard.

4.3 Results

4.3.1 Overexpression of *tHMG1* by integrating it into genome

Enzymatic activity of HMG-CoA reductase was assayed for CEN.PK113-7D and YIP-M0-03. Since HMG-CoA reductase consumes NADPH as a cofactor for reduction of HMG-CoA to mevalonate, the enzyme activities were tested based on the ability of cell extracts to oxidize NADPH in the presence of HMG-CoA as substrate. Overexpression of *tHMG1* increased the activity of this enzyme from 4.04 ± 0.49 in CEN.PK113-7D to 5.82 ± 0.29 U/(mg protein) in the YIP-M0-03 strain where U is defined as the nmol NADPH oxidized min^{-1}.

The effect of *tHMG1* overexpression on sesquiterpene biosynthesis was examined by measuring sesquiterpenes produced by the strains YIP-MV-07 and YIP-MC-13 in shake flask. These two strains produced approximately 40% more sesquiterpene compared to their corresponding control strains, YIP-0V-01 and YIP-0C-01, respectively (data not shown). Similar ratios for increase in the accumulation of both valencene and cubebol and increase in the enzyme activity of HMG-CoA reductase after *tHMG1* overexpression propose a flux controlling role and using the method of large deviations (Stephanopoulos et al., 1998) for calculation of the flux control coefficient a value of almost 1 is found for this enzyme in the mevalonate pathway.

4.3.2 Overexpression of *tHMG1* from plasmid

Since single copy integration of *tHMG1* in the genome led to only a 45% increase in the HMG-CoA reductase activity, we decided to further enhance the enzyme activity by expressing the *tHMG1* gene under control of a strong promoter in a high copy number plasmid. Therefore *tHMG1* and GFTpsC were cloned together into a pESC-URA plasmid under control of the strong inducible *GAL* promoters to make the plasmid pIP028. The plasmid pIP025 was also constructed as a control by cloning only GFTpsC into the pESC-URA plasmid. By transforming the YIP-M0-03 strain with the pIP028 plasmid it was possible to overexpress *tHMG1* both from the genome and a high copy number plasmid. The strains overexpressing *tHMG*1 were characterized in 5 L batch two-phase fermentations for both growth (Figure 4.2) and cubebol production (Figure 4.3). For all the strains overexpression of *tHMG1* resulted in a decrease in the specific growth rate (Figure 4.2) probably as a consequence of squalene accumulation which could be toxic for cells at high concentrations (Donald et al., 1997).

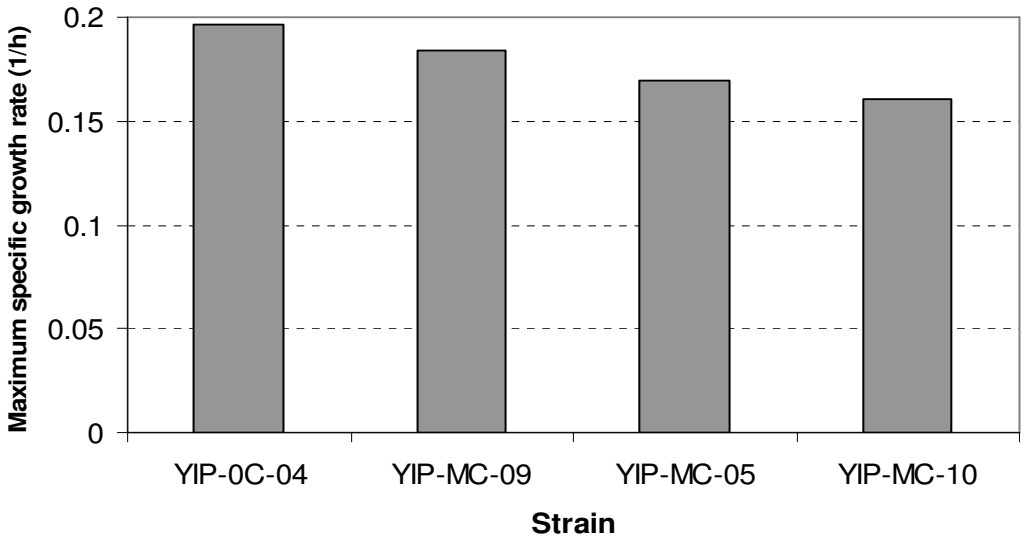

Figure 4.2. Effect of *tHMG1* overexpression on the maximum specific growth rate of yeast strains. Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose.

Overexpression of *tHMG1* led to increased final titers and yields of cubebol compared to the control strain, YIP-0C-04 (Table 4.3 and Figure 4.3). In Table 4.3 the total sesquiterpene concentration was calculated using the known percentage of cubebol in the mixture produced by the multi-product enzyme, cubebol synthase (Asadollahi et al., 2008). Overexpression of *tHMG1* from the genome or plasmid enhanced the cubebol production, but plasmid overexpression had a greater impact. However, simultaneous overexpression of *tHMG1* both from the plasmid and the genome in a single strain (YIP-MC-10) did not further improve cubebol production.

Comparison of the yield of cubebol on galactose and on ethanol showed that ethanol was a more efficient carbon source for cubebol production as the yields were higher on ethanol (Table 4.3).

Table 4.3. Final concentrations and yields of sesquiterpenes for the different yeast strains

Yeast strain	Cubebol (mg/L)	Total sesquiterpenes (excluding farnesol) (mg/L)	Farnesol (mg/L)	Yield of cubebol on biomass (mg/g DW)	Yield of cubebol on galactose (mg/g galactose)	Yield of cubebol on ethanol (mg/g ethanol)
YIP-0C-04	3.2	11.3		0.43	0.15	0.22
YIP-MC-09	5.1	18.2		0.52	0.17	0.55
YIP-MC-05	6.7	23.9		0.60	0.22	0.72
YIP-MC-10	5.3	18.9		0.58	0.17	0.46
YIP-MC-07	9.9	35.3	16.3	1.59	0.54	1.18
YIP-MC-08	9.0	32.1	18.4	1.44	0.54	1.06

Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose

4.3.3 Combining *tHMG1* overexpression and *ERG9* down-regulation

In an earlier study (Asadollahi et al., 2008) it was found that repression of *ERG9* in sesquiterpene producing yeast strains led to accumulation of the FPP derived compound, farnesol, indicating an increase in the intracellular pool of FPP. Besides farnesol, this strategy also improved the target sesquiterpene production with a more profound effect on patchoulol biosynthesis presumably because of higher activity of patchoulol synthase compared to valencene and cubebol synthases. Here we combined down-regulation of *ERG9* and overexpression of *tHMG1* in a cubebol producing yeast strain. Down-regulation of *ERG9* alone in a cubebol producing strain led to a 3-fold increase in cubebol titer but combination of *ERG9* down-regulation and *tHMG1* overexpression did not further improve cubebol production (Table 4.3 and Figure 4.3).

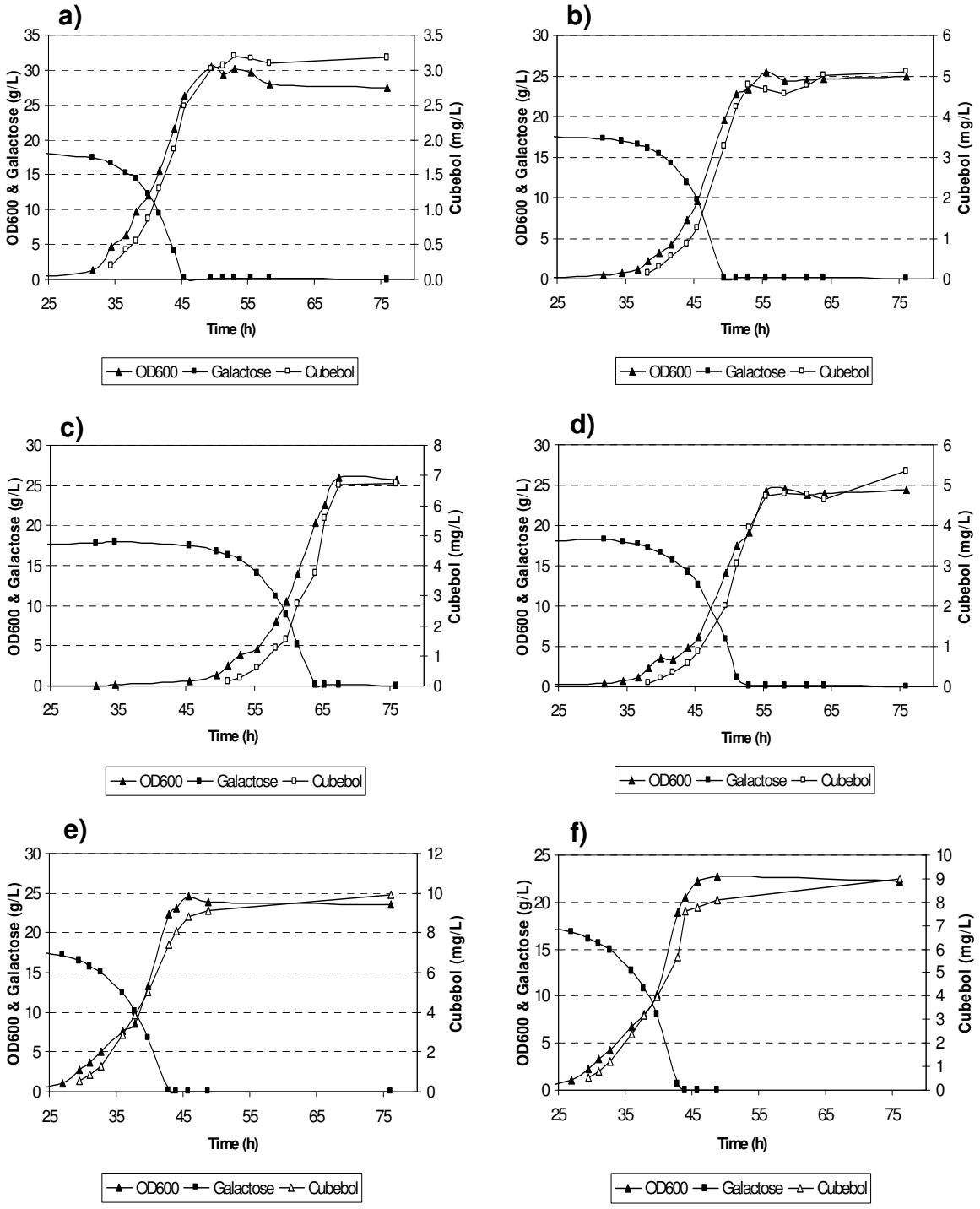

Figure 4.3. Cubebol, galactose and OD_{600} profiles as a function of time for the cubebol producing yeast strains (a) YIP-0C-04; (b) YIP-MC-09; (c) YIP-MC-05; (d) YIP-MC-10; (e) YIP-MC-07; (f) YIP-MC-08. Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose

4.3.4 Effect of *tHMG1* overexpression on ergosterol content of the cells

To assess the effect of *tHMG1* overexpression on ergosterol which is the end product of the pathway, the ergosterol content of *tHMG1* overexpressed strains were determined and compared with the control (YIP-0C-04) and WT (CEN.PK113-7D) strains (Figure 4.4). The increase in the ergosterol content of cells is not proportionally correlated with the increase in the HMG-CoA reductase activity and increase in the cubebol titer meaning that conversion of HMG-CoA to mevalonate is not the only rate controlling step in the complete ergosterol pathway and there are other regulatory sites downstream in the pathway towards ergosterol (Polakowski et al., 1998).

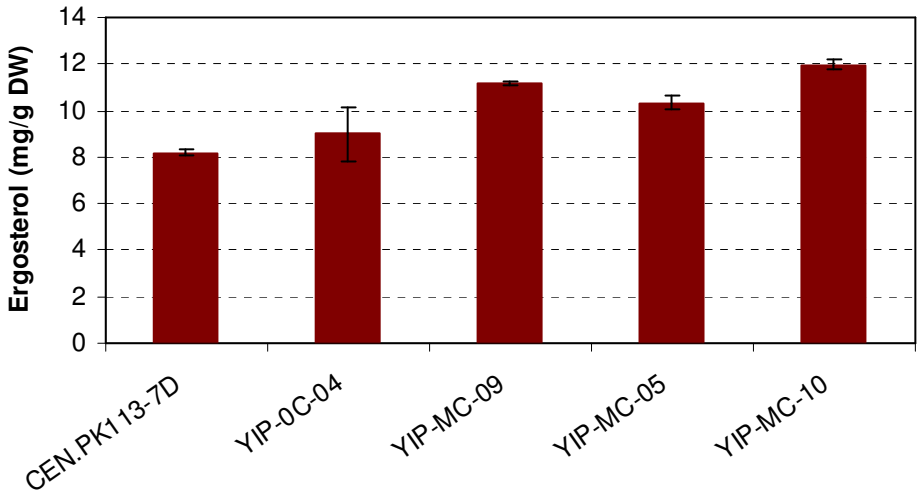

Figure 4.4. Influence of *tHMG1* overexpression on ergosterol content of yeast strains. Cells were grown in shake flasks containing 100 ml medium and 20 g/L galactose.

4.3.5 Ergosterol content of cells in the *ERG9* down-regulated strains

We previously observed reduced ergosterol content of cells upon down-regulation of *ERG9* by replacing its endogenous promoter with a regulated *MET3* promoter in the presence of methionine (Asadollahi et al., 2008). In this study, we also determined the ergosterol content of all *ERG9* down-regulated strains harboring the cubebol synthase gene or no sesquiterpene synthase (Figure 4.5). Surprisingly, we observed that ergosterol biosynthesis in the *ERG9* repressed strains is restored when cubebol synthase is expressed in this background strain both

on galactose and glucose whereas the YIP-M0-04 strain had lower ergosterol content as it was expected.

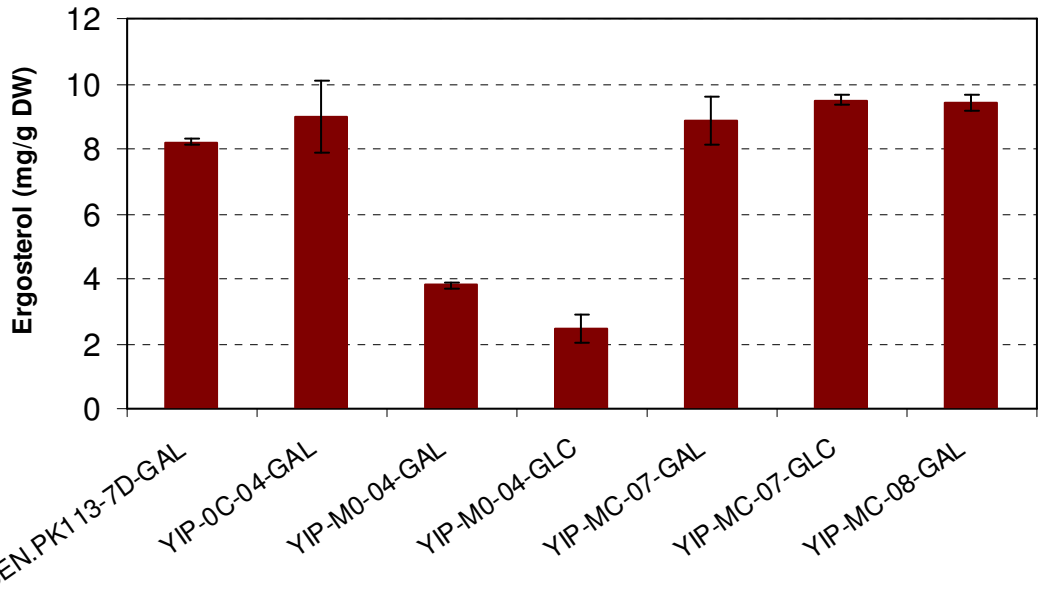

Figure 4.5. Influence of *ERG9* down-regulation and the combination of *ERG9* repression and *tHMG1* overexpression on ergosterol content of yeast strains. Cells were grown in shake flasks containing 100 ml medium and 20 g/L of either galactose (GAL) or glucose (GLC) as carbon sources. Repression of *ERG9* was induced by supplementing media with 2 mM methionine. YIP-M0-04 strain was also supplemented with 100 mg/L uracil as it had auxotrophy towards uracil.

4.3.6 Influence of *tHMG1* overexpression on squalene accumulation

Overexpression of *tHMG1* did not lead to a substantial increase in the ergosterol content indicating the existence of other regulatory steps in the pathway. One of these regulatory steps is conversion of squalene to squalene epoxide catalyzed by squalene epoxidase and therefore accumulation of squalene in the strains with higher activity of HMG-CoA reductase is expected (Polakowski et al., 1998). Extraction and measurement of squalene in the *tHMG1* overexpressed strains also revealed a dramatic change in squalene concentration from trace amounts in the WT and control strains to several milligrams per gram of biomass for the mutants (Figure 4.6).

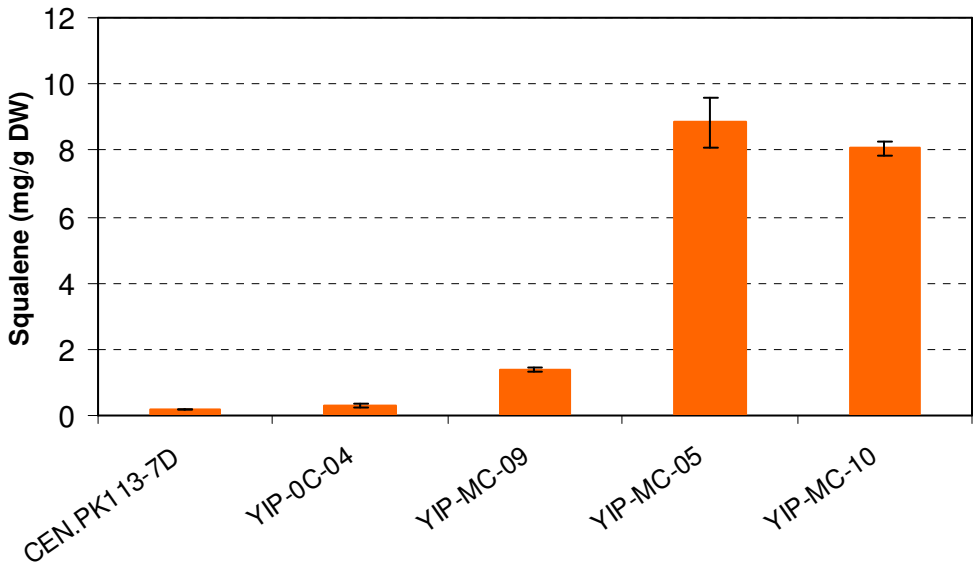

Figure 4.6. Influence of *tHMG1* overexpression on squalene accumulation in the yeast strains. Cells were grown in shake flasks containing 100 ml medium and 20 g/L galactose.

4.3.7 Effect of *ERG9* down-regulation on squalene accumulation

Quantification of ergosterol for the *ERG9* down-regulated strains showed unexpectedly high levels of ergosterol for these strains whenever cubebol synthase was also expressed. Therefore it would be interesting to see if expression of cubebol synthase in an *ERG9* repressed strain leads to accumulation of squalene as well. Figure 4.7 presents the squalene levels for the *ERG9* repressed strains. Again, to our surprise, we observed high squalene levels for the *ERG9* repressed strains upon expression of cubebol synthase but in contrary to the results from the ergosterol analysis, the squalene level was very low when the YIP-MC-07 strain was grown on glucose as carbon source (Figure 4.7).

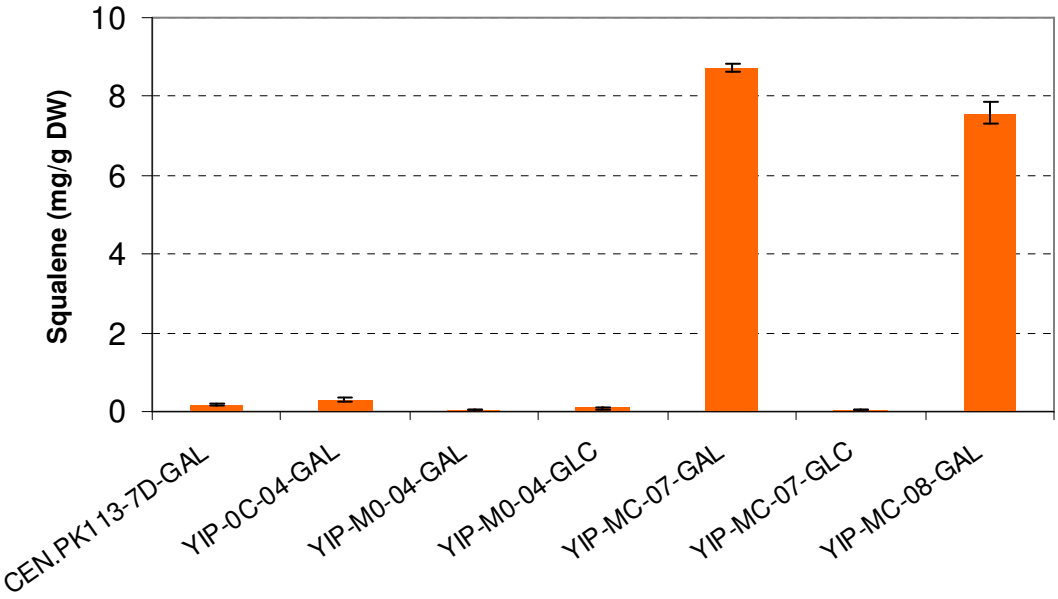

Figure 4.7. Influence of *ERG9* down-regulation and the combination of *ERG9* down-regulation and *tHMG1* overexpression on squalene accumulation in the different yeast strains. Cells were grown in shake flasks containing 100 ml medium and 20 g/L of either galactose (GAL) or glucose (GLC) as carbon sources. Repression of *ERG9* was induced by supplementing media with 2 mM methionine. YIP-M0-04 strain was also supplemented with 100 mg/L uracil since it had auxotrophy towards uracil.

4.4 Discussion

In this study, we deregulated the mevalonate pathway in the yeast *S. cerevisiae* in order to enhance the biosynthesis and also availability of FPP which is precursor for the biosynthesis of sesquiterpenes. Deregulation of the mevalonate pathway was accomplished by constructing strains overproducing HMG-CoA reductase which is responsible for the catalysis of the main flux controlling step in the mevalonate pathway. Overexpression of the truncated *HMG1* encoding the catalytic domain of HMG-CoA reductase was first done by integrating *tHMG1* into the genome under control of the strong constitutive *TPI1* promoter. This modification enhanced the activity of the enzyme by approximately 45% and increased the biosynthesis of valencene and cubebol by roughly 40% in shake flask experiments. The *tHMG1* gene was further overexpressed by cloning it into a high copy number plasmid under control of the strong inducible *GAL10* promoter. Overexpression of *tHMG1* from plasmid was more efficient and the final concentration of cubebol in batch fermenter reached to 6.7 mg/L whereas the cubebol concentration was 5.1 mg/L when *tHMG1* was overexpressed by genomic integration.

However, simultaneous overexpression of *tHMG1* from both the genome and high copy number plasmid did not further improve cubebol production.

In addition to enhancing sesquiterpene production, overexpression of *tHMG1* resulted in accumulation of squalene in the cells. Squalene accumulating organisms are scarce in the nature and the liver oil of deep-sea shark, which is traditionally used for squalene production (Donald et al., 1997), is one of the few exceptions with up to 60% squalene (Pietsch and Jaeger, 2007). Although the wild type strain contained trace amounts of squalene, the *tHMG1* overexpressed strains were able to accumulate up to 9 mg squalene/g DW. Accumulation of squalene as a result of *tHMG1* overexpression has been reported by other investigators as well (Donald et al., 1997; Polakowski et al., 1998) and proposes the reaction catalyzed by squalene epoxidase as a flux controlling step in the ergosterol biosynthetic pathway (Polakowski et al., 1998; Veen et al., 2003). Comparison of kinetic parameters of the enzymes squalene synthase and squalene epoxidase may explain why squalene expoxidase is a flux controlling step in the ergosterol pathway. The yeast squalene synthase has a K_m value of 2.5 μM for its substrate FPP (LoGrasso et al., 1993) and the wild type specific activity for this enzyme was found to be 460 pmol min^{-1} mg^{-1} (Jennings et al., 1991) whereas the reported numbers for the yeast squalene epoxidase are 13.5 μM and 32.1 pmol min^{-1} mg^{-1}, respectively (Satoh et al., 1993). The higher K_m value of squalene epoxidase for its substrate and lower specific activity of this enzyme suggest that squalene epoxidase has lower capacity than squalene synthase and this could lead to accumulation of squalene if the flux through the pathway exceeds a certain limit e.g. when HMG-CoA reductase is overproduced. Expression of cubebol synthase does not result in a sufficiently high drain of FPP from the sterol pathway to avoid squalene build up. Typical K_m values of sesquiterpene synthases for FPP are in the range of 0.4-10 μM (Picaud et al., 2005) which is comparable with that of yeast squalene synthase. However, the reported catalytic activities for sesquiterpene synthases are much smaller than the k_{cat} for the yeast squalene synthase. For instance, the k_{cat} values for amorpha-4,11-diene synthase from *Artemisia annua* L. vary from 3.4×10^{-4} to 15.4×10^{-3} s^{-1} depending on pH and the divalent metal ions (Picaud et al., 2005) and germacrene D synthase from *Zingiber officinale* has a k_{cat} value of 3.34×10^{-3} s^{-1} (Picaud et al., 2006) whereas squalene synthase has a k_{cat} value of 0.53 s^{-1} (LoGrasso et al., 1993). These low values indicate that the conversion of FPP to sesquiterpenes by sesquiterpene

synthases is less efficient than the condensation of two FPP molecules to squalene by squalene synthase.

Polakowski and coworkers (1998) noticed that the squalene content of HMG-CoA reductase overproduced yeast strains is not proportionally correlated with the activity of the enzyme since similar levels of squalene were produced for two strains that differed in the HMG-CoA reductase activity by a 16-fold factor. Since YIP-MC-05 and YIP-MC-10 strains accumulated close amounts of squalene it is likely that the squalene content is at inhibitory level and the strain YIP-MC-10 cannot support more flux through the pathway and this may explain why the strain YIP-MC-10 did not produce more cubebol than the strain YIP-MC-05.

Overexpression of *tHMG1* resulted in a reduction in the specific growth rate of yeast strains. This could be due to accumulation of squalene and its cytotoxic effects at high concentrations (Donald et al., 1997). Treatment of fungi with squalene epoxidase inhibitors such as terbinafine results in ergosterol depletion of fungal cell membrane and also high intracellular concentration of squalene which is believed to interfere with normal fungal membrane function and cell wall synthesis (Mukherjee et al., 2003; Liu et al., 2004). In contrary to the lower specific growth rate of squalene accumulating yeast strains observed by us and Donald et al. (1997), Veen et al. (2003) did not observe any adverse effect of squalene accumulation on cell growth.

FPP is a branch-point intermediate of the mevalonate pathway that serves as a substrate for several important enzymes. However most of the FPP is used for the biosynthesis of sterols and therefore minimizing the flux of FPP towards sterols would enhance available FPP for other enzymes of the branch-point including sesquiterpene synthases. We previously (Asadollahi et al., 2008) reduced the FPP utilization for sterols by replacing the endogenous *ERG9* promoter with a repressible *MET3* promoter. This strategy led to reduced ergosterol content of cells and enhanced sesquiterpene biosynthesis, but we also found that there was accumulation of farnesol as a major FPP derived by-product. Here, we combined overexpression of *tHMG1* and down-regulation of *ERG9* to both enhance the flux through the pathway and improve the availability of FPP for sesquiterpene synthases. Down-regulation of *ERG9* alone increased the cubebol titer more than 3-fold and farnesol was also accumulated. However, overexpression of *tHMG1* in this background did not further improve cubebol production. This does not accord with the results obtained by Ro and coworkers (2006) who reported a 2-fold increase in amorphadiene titer after down-regulation of *ERG9* in a *tHMG1* overexpressed yeast strain and a further 50%

increase after integration of a second copy of *tHMG1* in this background. Also for lycopene production in *C. utilis* it was shown that disruption of one of the copies of *ERG9* and overexpression of *tHMG1* resulted in improved lycopene production (Shimada et al., 1998).

Examination of the YIP-MC-07 and YIP-MC-08 strains for their sterol content revealed that these two strains were able to build up the same levels of ergosterol as the wild type strain during growth on both galactose and glucose as carbon source whereas the YIP-M0-04 strain had a lower ergosterol content. This means that expression of cubebol synthase restored the ergosterol biosynthesis in these strains. Surprisingly, these strains also accumulated a substantial amount of squalene comparable to the amounts accumulated by the *tHMG1* overexpressed strains, but only when they were grown on galactose as carbon source where the cubebol synthase is expressed. Since down-regulation of *ERG9* is expected to minimize the flux towards ergosterol and it was confirmed by determination of ergosterol content in the YIP-M0-04 strain, our findings arise the question whether this change is only a result of expressing cubebol synthase. We speculate that since ergosterol is vital for cell growth and there is a tight regulation in the pathway to maintain a certain amount of ergosterol in the cell one of the intermediates in the cubebol biosynthetic pathway is somehow able to react with FPP and make squalene thereby bypassing the native pathway for squalene and ergosterol biosyntheses. Since ergosterol has priority for the cell over sesquiterpenes, the first formed intermediates are rerouted to squalene and then to ergosterol. However, the mechanism by which squalene can be produced via cubebol synthase still remains to be addressed. The reason for the ergosterol level, but not the squalene level, being high on glucose could be due to some basal expression of cubebol synthase by the *GAL1* promoter in the presence of glucose. The expression of cubebol synthase in this case is low and the formed intermediates are channeled to ergosterol with no accumulation of squalene. Accumulation of squalene implies that the ergosterol content is high and squalene epoxidase is inhibited by feed back regulation triggered by ergosterol. This feed back regulation can also inhibit HMG-CoA reductase and thus increased expression of *tHMG1* cannot enhance the carbon flux towards FPP. In contrast to our results, in a very recent study on the production of amorphadiene by *ERG9* repressed yeast strains, addition of methionine to the medium reduced both ergosterol and squalene as expected (Paradise et al., 2008).

Comparison of cubebol yields in different phases of growth showed that ethanol was more efficient as carbon source than galactose. The higher yield on ethanol could be due to a higher cytosolic pool of acetyl-CoA when yeast grows on ethanol as carbon source. Also during growth on ethanol, the SNF1 protein kinase is activated, which subsequently inhibits the activity of acetyl-CoA carboxylase (Woods et al., 1994; Mitchelhill et al., 1994). This reduces the acetyl-CoA consumption for fatty acid biosynthesis and thus more acetyl-CoA can be directed to the mevalonate pathway. In addition, *ACS1,* which encodes acetyl-CoA synthetase is less repressed in the presence of ethanol compared to glucose (van den Berg et al., 1996) and probably galactose. Furthermore, metabolism of ethanol by its conversion to acetaldehyde and then acetate which is subsequently converted to acetyl-CoA is accompanied by generation of NADPH which is required as a cofactor for HMG-CoA reductase. Consistent with our finding, substantially higher yields of the polyketide 6-MSA has been reported during the yeast growth on ethanol (Wattanachaisaereekul et al., 2007).

In summary, the engineered yeast strains in this study produced up to approximately 10 mg/L cubebol or 51.6 mg/L of total sesquiterpenes. Taking into account the accumulated squalene and assuming that accumulated squalene originates from one of the intermediates of cubebol synthase, the yeast strains were able to produce roughly 110 mg/L sesquiterpene equivalents, and the yeast strains hereby represent a good platform for further development of an industrial process for production of sesquiterpenes.

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Chapter 5

Enhancing sesquiterpene production in *S. cerevisiae* through *in silico* driven metabolic engineering

5.1 Introduction

Metabolic engineering emerged over a decade ago as an independent scientific discipline following rapid developments in genetic engineering tools. This significantly facilitated rational design of cell factories in order to obtain desired cellular phenotypes (Nielsen, 2001). Since then, there have been numerous successful examples of metabolic engineering for improved production of endogenous or heterologous products, extension of substrate range used by microorganism, introduction of new catalytic activities for degradation of toxic compounds and manipulation of cell properties (Nielsen, 2001; Stephanopoulos et al., 1998). These remarkable accomplishments in most cases were achieved through alteration of gene expressions or introduction of new genes by taking the advantage of molecular biology tools.

Metabolic engineering of cell factories for heterologous production of isoprenoids is a prominent example showing how metabolic engineering strategies can be applied for improving a certain phenotype (for review see Maury et al., 2005). Application of metabolic engineering strategies

has allowed production of up to 480 mg/L amorphadiene (Newman et al., 2006), 102 mg/L lycopene (Yoon et al., 2006) and 503 mg/L β-carotene (Yoon et al., 2007) in engineered *E. coli* strains. There are also several studies on the engineering of the mevalonate pathway in yeast strains to enhance isoprenoid production (Yamano et al., 1994; Miura et al., 1998a; Miura et al., 1998b; Shimada et al., 1998; Jackson et al., 2003; Ro et al., 2006; Lindahl et al., 2006; Takahashi et al., 2007; Shiba et al., 2007; Asadollahi et al., 2008).

The metabolic engineering strategies applied to the yeast strains were mainly relying on the manipulation of the classical targets in the mevalonate pathway such as overexpression of *HMG1* (Shimada et al., 1998; Verwaal et al., 2007; Jackson et al., 2003; Ro et al., 2006), disruption or down-regulation of *ERG9* (Asadollahi et al., 2008; Ro et al., 2006; Shimada et al., 1998; Takahashi et al., 2007) and overexpression of *ERG20* (Ro et al., 2006). There are only a few studies on alteration of genes outside the mevalonate pathway e.g. engineering of the pyruvate dehydrogenase bypass (Shiba et al., 2007), overexpression of semi-dominant mutant allele of a global transcription factor regulating sterol biosynthesis in yeast, *upc2-1* (Ro et al., 2006; Jackson et al., 2003), and removing a phosphatase activity encoded by *DPP1* (Takahashi et al., 2007). In order to establish yeast as a platform for large scale production of isoprenoids, in addition to the genes directly involved in the mevalonate pathway, it is necessary to deregulate the pathway through manipulation of genes outside the pathway. However, this is a difficult task as it needs a comprehensive knowledge about all pathways and genes functioning in the cell and their interactions with each other, which is currently limited due to the immense complexity and non-linearity of interactions in the metabolic network and insufficient available information.

Nevertheless, the explosion of data generated by annotated genome sequences has encountered metabolic engineering with new challenges in the post-genomic era mainly concerning exploiting the wealth of information for improving cellular phenotypes (Alper and Stephanopoulos, 2004). As such, this abundance of data has led to reconstruction of several genome-scale metabolic networks (Covert et al., 2001) including *E. coli* (Edwards and Palsson, 2000; Reed et al., 2003), *Haemophilus influenzae* (Edwards and Palsson, 1999), and *S. cerevisiae* (Förster et al., 2003). Current genome scale models are, however, mainly stoichiometric and comprise a set of stoichiometric reactions to describe the biochemical reactions in the system (Patil et al., 2004). These genome scale models can be used to predict the cell behavior in certain conditions e.g. when a gene is disrupted by using constraint-based models such as flux balance

analysis (FBA) (Kauffman et al., 2003). This method is used for evaluating the feasible flux distributions in a metabolic network at steady-state conditions and assuming maximum growth as objective function. Adaptation of metabolic network for optimal growth is logical for wild type strains as a consequence of evolutionary pressure during million of years but may not hold for the mutant strains artificially constructed in the laboratory. Minimization of metabolic adjustments (MOMA) between a wild type and a knockout mutant could therefore be a more realistic objective function for assessing the flux distributions in the metabolic networks of deletion mutants (Segrè et al., 2002). The prediction power of FBA/MOMA simulations can effectively be used for metabolic engineering by formulating a bi-level programming framework for suggesting gene deletions leading to overproduction of a certain biochemical compound (Burgard et al., 2003).

In this study, we sought to assess the effect of gene deletions on the flux distributions in the stoichiometric model of *S. cerevisiae* (Förster et al., 2003) in order to identify target genes whose deletions can improve sesquiterpene production in yeast. OptGene (Patil et al., 2005) which is an extension of OptKnock (Burgard et al., 2003) was used as a modeling framework and MOMA as objective function to identify the gene knockouts leading to enhanced sesquiterpene production. OptGene uses a genetic algorithm method to search for the global optimal solution and is advantageous over OptKnock because it demands less computational time and allows optimization of non-linear objective functions (Patil et al., 2005).

5.2 Materials and methods

5.2.1 *In silico* computations

Conversion of FPP to sesquiterpenes catalyzed by sesquiterpene synthases was introduced into the stoichiometric model of *S. cerevisiae* (Förster et al., 2003). This model consisted of 1175 metabolic reactions and 584 unique metabolites.

The gene knockouts leading to enhanced sesquiterpene biosynthesis were found by using the OptGene algorithm (Patil et al., 2005) and MOMA as objective function. Thus, the knockouts leading to higher sesquiterpene yields were selected and further tested experimentally.

5.2.2 Strain construction

The plasmids pIP025 and pIP028 and also the strains YIP-0C-04 and YIP-MC-05 were described previously (chapter 4). In order to construct the strains YIP-00-07 and YIP-00-08, the CEN.MS1-10C T1 and CEN.PK113-13D were mated together on YPD plates. The next day some of the large colonies (the large colonies are more likely to be diploid) were picked and placed on an YPD master plate. After growing one day on this plate, the cells were replica plated onto a sporulating plate. After 3 days at 30 ºC, a small bit of cells from the sporulation plate was taken off with a sterile toothpick and mixed with 10 µl of sterile distilled water. 10 µl of 5 mg/ml Zymolyase was added to the mixture and incubated at room temperature for 10 min. The reaction was stopped by placing the samples on ice and adding 150 µl of sterile H_2O. Spores were dissected, separated with a glass needle under microscope, and grown on YPD medium for 3 days at 30 °C.

In the next step the tetrads were tested for the desired genotypes. Disruption of *URA3* was confirmed by growing the strains on SC (synthetic complete medium containing 2% glucose) and SC-ura (synthetic complete medium without uracil). Overexpression of *GDH2* was also verified by growing the strains on YPD plates supplemented with 300 mg/L G418.

To identify the mating type, the haploid strains on the dissection plate were mated with mating tester strains. Those plates were replica plated onto sporulation plates and incubated at 30 ºC for 3-4 days. The ability of spores to fluoresce under UV lamp at 302nm wavelength, made it possible to see which strain was originally *MAT a* and which was *MAT α*.

Deletion of *GDH1* in the strains was verified by colony PCR using the primers GGTTTGGGCAGGGAAATGTC and GTCCAATCAGCAGAGAGAAG. Thus, the strains YIP-00-07 and YIP-00-08 were obtained. Transformation of these strains with either pIP025 or pIP028 plasmid resulted in YIP-0C-05, YIP-MC-11 and YIP-0C-06 strains. Table 5.1 lists the strains used in this study.

5.2.3 Media for batch cultivations

A defined minimal medium as described by Verduyn et al. (1992) containing 20 g/L of galactose as the sole carbon source was used for all batch fermentations. The media had the following compositions: 5 g/L $(NH_4)_2SO_4$; 3 g/L KH_2PO_4; 0.5 g/L $MgSO_4.7H_2O$; 1 ml/L trace metal solution; 1 ml/L vitamin solution and 50 µl/L synperonic antifoam.

Galactose was autoclaved separately from the medium and subsequently added to the fermenter, as was the case for the vitamin solution that was added after filter sterilization.

5.2.4 Batch fermentations

Batch fermentations were carried out in well-controlled 5 L in-house manufactured glass bioreactors with a working volume of 4 L. The bioreactors were equipped with two disk-turbine impellers and 4 baffles to ensure proper mixing. The pH was controlled between 4.95 and 5.05 by automatic addition of 2M NaOH. The temperature was kept constant at 30 ºC. The air flow was 4 L/min (1 vvm) and was sterilized by filtration and the off gas passed through a condenser. Carbon dioxide and oxygen concentrations in the off-gas were determined by a Brüel & Kjær acoustic gas analyzer (Brüel & Kjær, Nærum, Denmark). Batch fermenters were inoculated to an initial OD_{600} of 0.02 from a liquid preculture. Three hundred milliliters of dodecane was added aseptically to the media at OD_{600} 0f 1 ± 0.1.

5.2.5 OD and dry weight determinations

The OD of samples was determined at 600 nm in duplicate by using a Hitachi U-1100 spectrophotometer. Dry weight measurement was achieved by using 0.45 µm pore-size nitrocellulose filters (Sartorius AG, Göttingen, Germany) according to the method described by Dynesen et al. (1998).

5.2.6 Analysis of galactose and extracellular metabolites

Determination of galactose and extracellular metabolites was performed as described previously (Asadollahi et al., 2008).

5.2.7 Analysis of sesquiterpenes in the organic layer

Samples from organic layer were centrifuged for 5 min at 3500 rpm and subsequently analyzed by GC-MS to determine the level of sesquiterpenes during the course of fermentation as described (Asadollahi et al., 2008).

Table 5.1. Strains used in this study

Strain	Genotype	Plasmid	Plasmid description	Reference
YIP-00-03 (CEN.PK113-5D)	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	none		Peter Kötter[a]
CEN.PK113-13D	*MATα MAL2-8*^{c} *SUC2 ura3-52*			Peter Kötter[a]
CEN.MS1-10C T1	*MAT***a** *MAL2-8*^{c} *SUC2* *gdh1(209,1308)::loxP* *gdh2::*P_{PGK}*-GDH2-KanMX3*	none		Santos et al., 2003
YIP-00-08	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52* *gdh1(209,1308)::loxP*	none		This study
YIP-00-07	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52* *gdh1(209,1308)::loxP* *gdh2::*P_{PGK}*-GDH2-KanMX3*	none		This study
YIP-0C-04	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP025	pESC-URA 2µ *URA3* P_{GAL1}- GFTpsC	Chapter 4
YIP-MC-05	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP028	pESC-URA 2µ *URA3* P_{GAL1}-GFTpsC P_{GAL10}*-tHMG1*	Chapter 4
YIP-0C-06	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52* *gdh1(209,1308)::loxP*	pIP025	pESC-URA 2µ *URA3* P_{GAL1}- GFTpsC	This study
YIP-0C-05	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52* *gdh1(209,1308)::loxP* *gdh2::*P_{PGK}*-GDH2-KanMX3*	pIP025	pESC-URA 2µ *URA3* P_{GAL1}- GFTpsC	This study
YIP-MC-11	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52* *gdh1(209,1308)::loxP* *gdh2::*P_{PGK}*-GDH2-KanMX3*	pIP028	pESC-URA 2µ *URA3* P_{GAL1}-GFTpsC P_{GAL10}*-tHMG1*	This study

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5.3 Results

5.3.1 *In silico* metabolic engineering

The reaction for conversion of the precursor FPP to sesquiterpenes was added to the original genome-scale model of *S. cerevisiae* (Förster et al., 2003) and the intracellular fluxes of the resulting model were calculated for the knockout mutants subject to MOMA using OptGene (Patil et al., 2005) to find the genes whose deletion could increase the flux towards sesquiterpenes. Deletion of *GDH1* coding glutamate dehydrogenase turned out to be the best target gene for the further experimental work.

Glutamate dehydrogenase encoded by *GDH1* is involved in ammonium metabolism in yeast. Once imported in the cell, ammonium is incorporated into amino group of glutamate and amide group of glutamine which serve as nitrogen source for 85% and 15% of the total nitrogen containing compounds in the cell, respectively (Magasanik and Kaiser, 2002). There are two metabolic routes for the biosynthesis of glutamate as the main source of nitrogen in the cell (Figure 5.1) (ter Schure et al., 2000). In the first strategy which is the major route in *S. cerevisiae*, ammonium reacts with α-ketoglutarate to form glutamate. This reaction is catalyzed by NADPH-dependent glutamate dehydrogenase encoded by *GDH1* (Moye et al., 1985). The other pathway which is a two step process (GS-GOGAT) is mediated by glutamine synthetase (GS) and glutamate synthase (GOGAT) encoded by *GLN1* and *GLT1*, respectively (Magasanik, 2003).

There are two other glutamate dehydrogenases in *S. cerevisiae* encoded by *GDH2* and *GDH3*. In contrary to *GDH1,* which uses NADPH as cofactor, *GDH2* is NADH-dependent. However, Gdh2p has much lower activity than Gdh1p in normal cell when ammonium is used as the sole nitrogen source (Nissen et al., 1997). *GDH3* also codes an NADPH-dependent glutamate dehydrogenase activity and it was shown that the cells lacking both *GDH1* and *GLT1* are still able to grow and are not completely glutamate auxotroph whereas the triple *GDH1 GDH3 GLT1* mutants were glutamate auxotroph suggesting a physiological role for *GDH3*. Nevertheless, simultaneous deletion of *GDH1* and *GDH3* did not affect the cell phenotype compared to the single *GDH1* deletion which implies NADPH-dependent *GDH1* and the GS-GOGAT pathway are the major routes for ammonium assimilation (Avendaño et al., 1997).

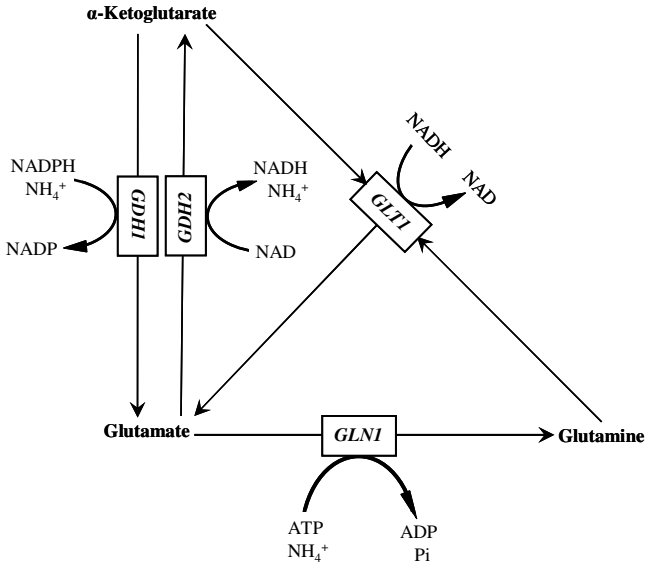

Figure 5.1. Ammonium assimilation pathway in *S. cerevisiae*

A substantial amount of NADPH in the cell is used as cofactor for the reaction catalyzed by NADPH-dependent glutamate dehydrogenase (*GDH1*) (dos Santos et al., 2003). Therefore, if *GDH1* is deleted there will be more NADPH available for other NADPH requiring reactions in the cell. In the third step of the mevalonate pathway in yeast, HMG-CoA is converted to mevalonate by the action of HMG-CoA reductase. This reaction is considered as a major flux controlling step in the mevalonate pathway (Veen and Lang, 2004) and requires NADPH as cofactor. Deletion of *GDH1* could be beneficial for this reaction as there will be more accessible NADPH for the HMG-CoA reductase and therefore deletion of *GDH1* suggested by *in silico* metabolic engineering makes biologically sense. However, since disruption of *GDH1* impairs the ammonia utilization, overexpression of the NADH-dependent glutamate dehydrogenase, *GDH2*, was also considered in this study.

5.3.2 Effect of alterations on specific growth rates and yields

The strains were grown in 5 L batch two phase fermenters. Table 5.2 summarizes the specific growth rates and yields of ethanol and glycerol on galactose for the characterized strains. Deletion of *GDH1* severely affected the specific growth rate of yeast strains. However, overexpression of *GDH2* restored the growth to some extent. Overexpression of *tHMG1* further improved the specific growth rate which is in contrary to our previous observation that

overexpression of *tHMG1* led to lower specific growth rates (Chapter 4). Manipulation of the nitrogen metabolism did not affect the yield of ethanol substantially but there was a marked decrease in the yield of glycerol.

Table 5.2. Maximum specific growth rates on galactose (μ_{max}), yields of ethanol on galactose ($Y_{S/EtOH}$), and yields of glycerol on galactose ($Y_{S/Glycerol}$) for the characterized strains

Strain	μ_{max} (h^{-1})	$Y_{S/EtOH}$ (g/g)	$Y_{S/Glycerol}$ (g/g)
YIP-0C-04	0.20	0.138	0.015
YIP-0C-06	0.07	0.133	0.006
YIP-0C-05	0.13	0.142	0.008
YIP-MC-11	0.15	0.120	0.013

5.3.3 Sesquiterpene production

The strains manipulated in the ammonium assimilation pathway were characterized for sesquiterpene production in 5 L two phase batch fermenters (Figure 5.2). Table 5.3 lists the final titer and yield of sesquiterpenes for the cubebol producing yeast strains. In Table 5.3 total sesquiterpenes were calculated using the known percentage of cubebol in the mixture produced by the multi-product enzyme, cubebol synthase (Asadollahi et al., 2008). Deletion of *GDH1* as was predicted by *in silico* modeling increased cubebol production substantially. Overexpression of *GDH2* did not further enhance cubebol production but as mentioned above it resulted in an increased specific growth rate in the YIP-0C-05 compared to the YIP-0C-06 strain. Overproduction of the catalytic domain of HMG-CoA reductase (YIP-MC-11 strain) further improved cubebol production as it was expected.

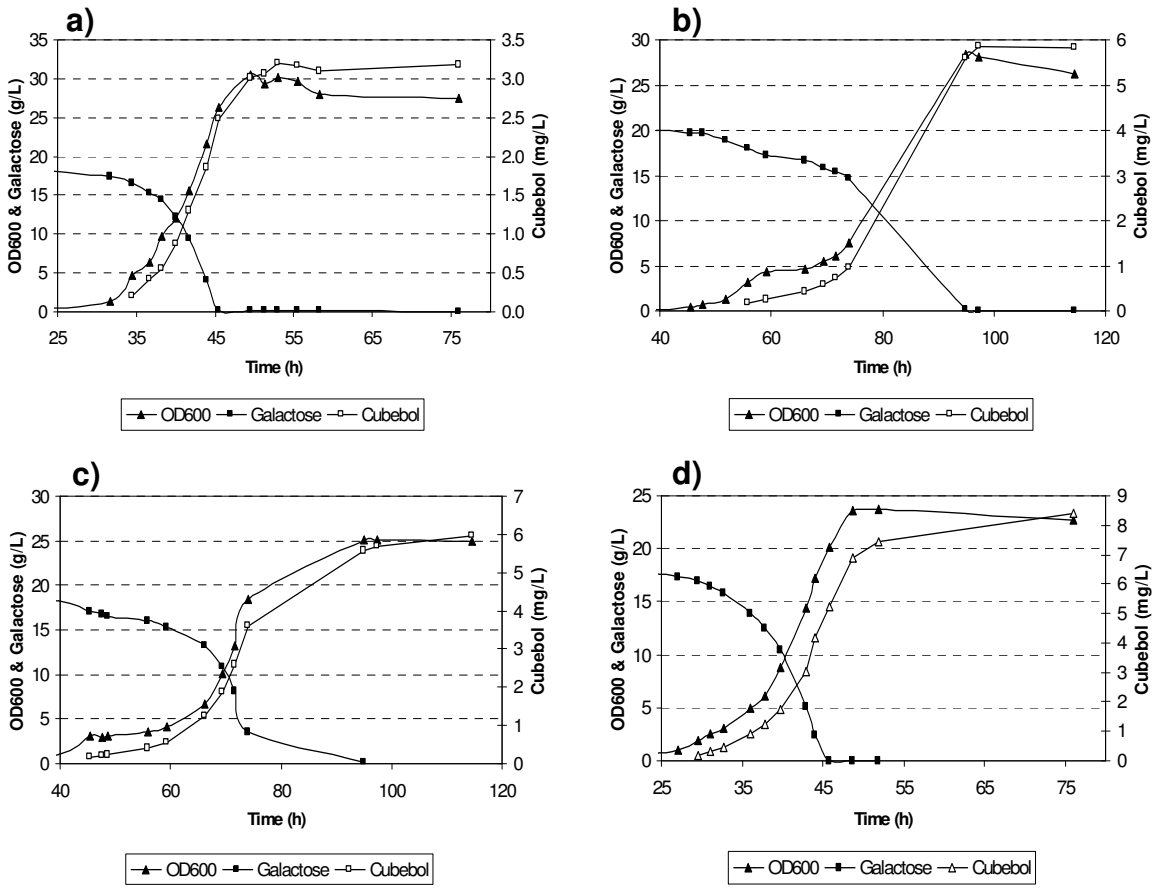

Figure 5.2. Cubebol, galactose and OD_{600} profiles as a function of time for the cubebol producing yeast strains (a) YIP-0C-04; (b) YIP-0C-06; (c) YIP-0C-05; (d) YIP-MC-11
Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose.

Table 5.3. Final concentrations and yields of sesquiterpenes for the different yeast strains

Yeast strain	Cubebol (mg/L)	Total sesquiterpenes (mg/L)	Yield of cubebol on biomass (mg/g DW)	Yield of cubebol on galactose (mg/g galactose)
YIP-0C-04	3.2	11.3	0.43	0.15
YIP-0C-06	5.9	20.9	0.83	0.26
YIP-0C-05	6.0	21.3	0.81	0.28
YIP-MC-11	8.4	30.1	0.83	0.24

Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose

5.4 Discussion

Metabolic engineering of cell factories in order to improve a cellular phenotype e.g. overproduction of a biochemical compound often requires manipulation of several genes in the cell. However, as the metabolic pathways are highly inter-connected, manipulation of a gene will often have secondary effects on other pathways and subsequently on the cell behavior. Therefore there has been a paradigm shift in metabolic engineering from focusing only on one gene related with the pathway of interest to the entire metabolic networks. However, because of the complicated regulation, lack of information on the kinetics of enzymes and little information on the mechanism of interaction of pathways, it is difficult to predict the minimum genetic modifications in order to exploit the maximum capacity of cell for a desired phenotype. Despite their limited predictive power (Gombert and Nielsen, 2000), genome scale models that are primarily stoichiometric models, can be used to suggest required changes in the genotype for a desired phenotype. This strategy has been used with success for identifying new target genes for heterologous production of lycopene in *E. coli* (Alper and Stephanopoulos, 2004; Alper et al., 2005).

In this study, we used the genome-scale reconstructed metabolic network of *S. cerevisiae* (Förster et al., 2003) to identify new target genes for improved production of cubebol in yeast. Disruption of NADPH-dependent glutamate dehydrogenase encoded by *GDH1* was identified as the best target. Deletion of this gene will enhance the available NADPH in the cytosol for other NADPH requiring reactions, including HMG-CoA reductase. In addition to disruption of *GDH1*, overexpression of the NADH-dependent glutamate dehydrogenase encoded by *GDH2* was also pursued to restore efficient utilization of ammonium by cells. Manipulation of the ammonium assimilation pathway has been carried out for the purpose of improving ethanol production through reducing glycerol formation, the main by-product in anaerobic yeast fermentation. Thus, deletion of *GDH1* increased the ethanol yield by 8% and reduced the glycerol yield by 38%. The increased NADH consumption and ATP formation by the alternative GS-GOGAT pathway was the reason for these changes (Nissen et al., 2000). Likewise, this strategy enhanced ethanol production on xylose (Roca et al., 2003). Deletion of glutamate dehydrogenase (*gdhA*) in lycopene producing *E. coli* also led to a 13% improvement in lycopene accumulation (Alper and Stephanopoulos, 2004; Alper et al., 2005).

Deletion of *GDH1* in the current study led to an approximately 85% increase in the final cubebol titer. However, deletion of this gene also caused a significant decrease in the maximum specific growth rate. This is in consistence with earlier reports on the reduced growth rate of yeast strains on glucose as a consequence of *GDH1* deletion in both anaerobic (Nissen et al., 2000) and aerobic (dos Santos et al., 2003) conditions. Overexpression of *GDH2* did not show a further effect on the final cubebol titer but the YIP-0C-05 strain had a higher maximum specific growth rate compared to the YIP-0C-06 strain. However, the maximum specific growth rate of the YIP-0C-05 strain was still lower than that of YIP-0C-04 while in other studies overexpression of *GDH2* completely restored the specific growth rate on glucose to the wild type level (Nissen et al., 2000; dos Santos et al., 2003). Although overexpression of *GDH2* enables cells to utilize ammonium as nitrogen source more efficiently, the modification of ammonium assimilation pathway changes the NADPH/$NADP^+$ ratio significantly. This could in turn have secondary effects such as channeling isocitrate into the glyoxylate cycle (Satrustegui et al., 1983) or reduced flux through the pentose-phosphate pathway (dos Santos et al., 2003).

The glycerol yields for both YIP-0C-06 and YIP-0C-05 were also significantly lower than that of YIP-0C-04 strain but there was not a marked change in ethanol yields. The surplus NADH in the cell is reoxidized to NAD^+ to avoid imbalances in the NAD^+/NADH ratio. Since deletion of *GDH1* reroutes the formation of glutamate to the other NADH-dependent pathways there will be less free NADH and subsequently less glycerol formation (Nissen et al., 2000). Deletion of *GDH1* in the YIP-0C-06 strain leads to the formation of glutamate via the GS-GOGAT pathway accompanied by higher ATP consumption, which is presumably compensated by more ethanol formation (Nissen et al., 2000). However, in aerobic conditions the oxidative phosphorylation pathway is active and the slightly higher ATP consumption for growth does therefore not have a pronounced effect (dos Santos et al., 2003).

Formation of 100 g yeast biomass growing on ammonium as nitrogen source requires 931 mmol of NADPH (Bruinenberg et al., 1983). In this study, the highest yield of cubebol on biomass was 0.83 mg/g DW (2.96 mg total sesquiterpenes/ g DW). Assuming the molecular weight of 222 for all sesquiterpenes formed by cubebol synthase this yield is equivalent to 0.0133 mmol total sesquiterpenes/g DW. Since biosynthesis of each mmol sesquiterpene requires 6 mmol NADPH by HMG-CoA reductase as cofactor the highest NADPH requirement is 8 mmol/100 g DW. This is less than 1% of total NADPH requirement in the cell and therefore the question arises how

enhancing NADPH availability leads to improved sesquiterpene production. We speculate that the higher available pool of NADPH would thermodynamically favor the NADPH consuming reactions towards NADPH utilization and $NADP^+$ generation to balance NADPH/$NADP^+$ ratio. However, if the flux towards the mevalonate pathway is increased, at some point the availability of NADPH could also be a limiting factor for HMG-CoA reductase.

Earlier work showed a lower specific growth rate when *tHMG1* was overexpressed, presumably caused by accumulation of squalene (Chapter 4). By contrast, in this study the YIP-MC-11 had a higher maximum specific growth rate than the YIP-0C-05 strain. We speculate that since overexpression of *tHMG1* increases the NADPH demand it could be beneficial for the cells with the modified ammonium utilization as it may lead to a reduction in the high NADPH/$NADP^+$ ratio.

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Chapter 6

Metabolic engineering of *S. cerevisiae* for lycopene production

6.1 Introduction

Carotenoids comprise one of the largest and most diverse groups of naturally occurring pigments (Armstrong, 1997) and are responsible for the beautiful colors of many fruits and vegetables such as reds of tomatoes and oranges of carrots. Carotenoids are found in all photosynthetic organisms as well as in some non-photosynthetic bacteria and fungi (Hirschberg et al., 1997). Carotenoids play a variety of essential biological roles including antioxidant defense against photooxidation, accessory light harvesting components in photosynthesis, and protection against reactive oxygen species. Carotenoids are commercially used as food colorants, feed supplements in poultry and fish farming, and nutraceuticals (Lee and Schmidt-Dannert, 2002; Schmidt-Dannert et al., 2006). Of about 700 identified carotenoids in various organisms (Hornero-Méndez and Britton, 2002) only a few are currently produced in high quantities (Schmidt-Dannert et al., 2006). Although the initial large-scale processes for the production of carotenoids were based on native carotenoid producing organisms, the cheaper production by chemical synthesis outperformed the biological processes (Nicolás-Molina et al., 2008; Ausich, 1997). However, due to new findings about the

potential applications of carotenoids in preventing the onset of many diseases (Rao and Rao, 2007; Fraser and Bramley, 2004) and an increasing demand for carotenoids, there has been a renewed attention for biotechnological production of carotenoids. In particular, isolation and characterization of more than 150 carotenogenic genes from several organisms (Lee and Schmidt-Dannert, 2002; Schmidt-Dannert, 2000) along with the rapid advances in recombinant DNA technologies have eased heterologous production of carotenoids in non-carotenogenic microorganisms.

One of the key properties of carotenoids is their ability to quench singlet oxygen which is highly dependent on the number of double conjugated bonds in the molecule. Lycopene with 11 double conjugated bonds is the most effective carotenoid in this regard and its *in vitro* quenching rate constant is more than 2-fold higher than that of β-carotene (Di Mascio et al., 1989).

Tomato is one of the most widely used natural sources of lycopene, but the lycopene concentration in tomato (10-30 mg/kg) is still not high enough to support a viable industrial source for lycopene production (Kerr et al., 2004). Therefore there have been several attempts to establish microbial production of lycopene in particular in non-carotenogenic microorganisms (Maury et al., 2005). In most of the studies on heterologous biosynthesis of lycopene, *E. coli* was used as the microbial host. However, there are also some reports on heterologous expression of carotenogenic genes in *S. cerevisiae* (Yamano et al., 1994) and *C. utilis* (Miura et al., 1998a; Miura et al., 1998b; Shimada et al., 1998) for lycopene production. In addition to its potential applications, lycopene can serve as a tool in metabolic engineering studies for high-throughput screening of overproducing mutants due to its strong red color (Alper et al., 2005; Kang et al., 2005; Klein-Marcuschamer et al., 2007; Alper and Stephanopoulos, 2007). Furthermore, lycopene is an intermediate for other carotenoids and hence a mutant overproducer of lycopene can be used as a platform for the biosynthesis of other carotenoids as well.

Previously, we deregulated the mevalonate pathway in *S. cerevisiae* in order to produce various plant sesquiterpenes (Asadollahi et al., 2008; Chapter 4; Chapter 5). Here, we studied heterologous production of lycopene in engineered yeast strains. The strains were engineered for overproduction of the catalytic domain of the enzyme catalyzing the flux controlling step in the mevalonate pathway, HMG-CoA reductase, and down-regulation of *ERG9* to minimize the flux towards ergosterol.

In order to construct lycopene producing yeast strains, we expressed the codon optimized *crtE*, *crtB*, and *crtI* genes of the bacterium *Erwinia herbicola* encoding geranylgeranyl diphosphate synthase, phytoene synthase and phytoene desaturase, respectively. The constructed strains were characterized in batch fermenters for lycopene production.

6.2 Materials and methods

6.2.1 Plasmid construction

The codon optimized *crtE*, *crtB*, and *crtI* genes from *E. herbicola* were synthesized (Entelechon GmbH, Regensburg, Germany) and cloned into pCR®4-TOPO cloning vector (Invitrogen). The sequences of original *crtE*, *crtB*, and *crtI* genes from *E. herbicola* and the codon optimized genes are shown in Appendix A. Cloning of *crtE*, *crtB*, and *crtI* genes into pCR®4-TOPO cloning vector led to the plasmids pIP033, pIP034, and pIP035, respectively. The plasmid pIP036 was constructed by cloning *crtE* in a pESC-URA vector (Stratagene) under control of *GAL10* promoter using *Sac*I and *Not*I restriction sites. The plasmid pIP037 was constructed by cloning *crtI* in a pESC-HIS vector (Stratagene) under control of *GAL10* promoter using *Spe*I and *Sac*I restriction sites. Cloning of *crtB* under control of *GAL1* promoter in the plasmid pIP037 using *Sal*I and *Xho*I restriction sites resulted in the plasmid pIP038. The plasmid pIP039 was obtained by combining the pIP036 and pIP038 plasmids using gap repair technique. The piece of plasmid pIP038 containing *crtB* and *crtI* genes including their promoters and terminators was amplified using pURA_GAP_F and pURA_GAP_R primers (Table 6.1). The PCR conditions were in accordance with the Phusion™ Hot Start High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland). The pIP036 plasmid was digested with *Mfe*I restriction enzyme in parallel. The PCR product and the digested expression vector were separated by gel electrophoresis and gel purified using the QIAEX® II Gel extraction kit (Qiagen, Hilden, Germany). Homologous recombination of the PCR product into the digested expression vector after transformation of the strain CEN.PK113-5D with both fragments resulted in the plasmid pIP039.

The plasmid pIP042 was obtained by cloning *crtB* in the plasmid pIP036 under control of *GAL1* promoter using *Sal*I and *Xho*I restriction sites.

Overexpression of the catalytic domain of HMG-CoA reductase (*tHMG1*) was performed using gap repair technique. The *tHMG1* gene was amplified from the genomic DNA of *S. cerevisiae* CEN.PK113-7D using the primers GAP_HMG1_F and GAP_HMG1_R (Table 6.1). The

truncated coding region has an engineered Met start codon and then continues with the natural coding region from codon 531 (Asp) to the natural stop codon. The plasmid pIP037 was digested with *Bam*HI and *Xma*I restriction enzymes. Homologous recombination of the PCR product into the digested pIP037 plasmid after transformation of the strain CEN.PK113-11C with both fragments resulted in the pIP043 plasmid.

The plasmid pIP044 was obtained by combining the pIP042 and pIP043 plasmids using gap repair technique. The piece of plasmid pIP043 containing *tHMG1* and *crtI* genes including their promoters and terminators was amplified using pURA_GAP_F and pURA_GAP_R primers (Table 6.1). The pIP042 plasmid was digested with *Mfe*I restriction enzyme in parallel. Homologous recombination of the PCR product into the digested expression vector after transformation of CEN.PK113-5D with both fragments resulted in the pIP044 plasmid.

The pIP041 plasmid was constructed using gap repair technique. The red fluorophore used in this study was a monomeric version of DsRed (mRFP1) (Campbell et al., 2002) and was amplified from the pWJ1350 plasmid (Lisby et al., 2003) using the RFP_crtI_F and RFP_R primers (Table 6.1). The primers were designed so that after gap repair the stop codon of *crtI* gene was removed and a linker including 3 glycine amino acids was introduced between *crtI* and mRFP1 (The bold underlined sequence in RFP_crtI_F primer). In parallel, the plasmid pIP038 was digested with *Pac*I and *Sac*I restriction enzymes. Homologous recombination of the PCR product into the digested pIP038 plasmid after transformation of CEN.PK113-11C with both fragments resulted in the pIP041 plasmid.

Table 6.2 and Table 6.3 list the plasmids and strains used in this study, respectively.

Table 6.1. Primers used for constructing the plasmids

Primer	Sequence
pURA_GAP_F	cagcactacccttagctgttctatatgctgccactcctACGCAAACCGCCTCTCCCCG
pURA_GAP_R	aggaaatgatagcattgaaggatgagactaatccaattaTCGGTGCGGGCCTCTTCGC
GAP_HMG1_F	actttaacgtcaaggagaaaaaaccccggatccACTATGGACCAATTGGTGAAAACTG
GAP_HMG1_R	ttcttcggaaatcaacttctgttccatgtcgacgccTTAGGATTTAATGCAGGTGACGGAC
RFP_crtI_F	tgctagtctgatgatcgaggatttgcaggagctc**GGTGGTGGT**ATGGCCTCCTCCGAGGA
RFP_R	gacaagccgacaaccttgattggagacttgaccaaacctctggcgaagaattgttaattaaTTAGGCGCCGG TGGAGT

Lower case letters are overhangs for homologous recombination. Upper case letters are the actual primer sequences. The underlined bold sequence shows the sequence coding for 3 glycine amino acid linker between *crtI* and mRFP1 genes.

Table 6.2. Plasmids used in this study

Plasmid	Description	Reference
pIP033	pCR®4-TOPO *crtE*	Entelechon GmbH
pIP034	pCR®4-TOPO *crtB*	Entelechon GmbH
pIP035	pCR®4-TOPO *crtI*	Entelechon GmbH
pIP036	pESC-URA 2µ *URA3* P_{GAL10}-*crtE*	This study
pIP037	pESC-HIS 2µ HIS*3* P_{GAL10}-*crtI*	This study
pIP038	pESC-HIS 2µ HIS*3* P_{GAL1}-*crtB* P_{GAL10}-*crtI*	This study
pIP039	pESC-URA 2µ *URA3* P_{GAL10}-*crtE* P_{GAL1}-*crtB* P_{GAL10}-*crtI*	This study
pIP041	pESC-HIS 2µ HIS*3* P_{GAL1}-*crtB* P_{GAL10}-*crtI-mRFP1*	This study
pIP042	pESC-URA 2µ *URA3* P_{GAL10}-*crtE* P_{GAL1}-*crtB*	This study
pIP043	pESC-HIS 2µ HIS*3* P_{GAL1}-*tHMG1* P_{GAL10}-*crtI*	This study
pIP044	pESC-URA 2µ *URA3* P_{GAL10}-*crtE* P_{GAL1}-*crtB* P_{GAL10}-*crtI* P_{GAL1}-*tHMG1*	This study

Table 6.3. Strains used in this study

Strain	Genotype	Plasmid	Reference
YIP-00-03 (CEN.PK113-5D)	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	none	Peter Kötter[a]
CEN.PK113-11C	*MAT***a** *MAL2-8*^{c} *SUC2 his3-*Δ*1 ura3-52*	none	Peter Kötter[a]
YIP-M0-04	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	none	Asadollahi et al., 2008
YIP-M0-03	*MAT***a** *MAL2-8*^{c} *SUC2, ura3 ::*P_{TPI1}*-tHMG1*	none	Chapter 4
YIP-0L-02	*MAT***a** *MAL2-8*^{c} *SUC2 his3-*Δ*1 ura3-52*	pIP036 & pIP038	This study
YIP-0L-03	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP039	This study
YIP-ML-03	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	pIP039	This study
YIP-ML-04	*MAT***a** *MAL2-8*^{c} *SUC2, ura3 ::*P_{TPI1}*-tHMG1*	pIP039	This study
YIP-0L-05	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP042	This study
YIP-ML-05	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	pIP042	This study
YIP-ML-06	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52*	pIP044	This study
YIP-ML-07	*MAT***a** *MAL2-8*^{c} *SUC2 ura3-52, erg9 ::*P_{MET3}*-ERG9*	pIP044	This study
YIP-ML-08	*MAT***a** *MAL2-8*^{c} *SUC2, ura3 ::*P_{TPI1}*-tHMG1*	pIP044	This study
YIP-0L-06	*MAT***a** *MAL2-8*^{c} *SUC2 his3-*Δ*1 ura3-52*	pIP036 & pIP041	This study

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6.2.2 Media for batch cultivations

A defined minimal medium as described by Verduyn et al. (1992) containing 20 g/L of galactose as the sole carbon source was used for all batch fermentations. The media had the following compositions: 5 g/L $(NH_4)_2SO_4$; 3 g/L KH_2PO_4; 0.5 g/L $MgSO_4.7H_2O$; 1 ml/L trace metal solution; 1 ml/L vitamin solution and 50 µl/L synperonic antifoam.

Galactose was autoclaved separately from the medium and subsequently added to the fermenter, as was the case for the vitamin solution that was added after filter sterilization.

6.2.3 Batch fermentations

Batch fermentations were carried out in well-controlled 5 L in-house manufactured glass bioreactors with a working volume of 4 L. The bioreactors were equipped with two disk-turbine impellers and 4 baffles to ensure proper mixing. The pH was controlled between 4.95 and 5.05 by automatic addition of 2M NaOH. The temperature was kept constant at 30 ºC. The air flow was 4 L/min (1 vvm) and was sterilized by filtration and the off gas passed through a condenser. Carbon dioxide and oxygen concentrations in the off-gas were determined by a Brüel & Kjær acoustic gas analyzer (Brüel & Kjær, Nærum, Denmark). Batch fermenters were inoculated to an initial OD_{600} of 0.02 from a liquid preculture.

6.2.4 OD and dry weight determinations

The OD of samples was determined at 600 nm in duplicate by using a Hitachi U-1100 spectrophotometer. Dry weight measurement was achieved by using 0.45 µm pore-size nitrocellulose filters (Sartorius AG, Göttingen, Germany) according to the method described by Dynesen et al. (1998).

6.2.5 Analysis of galactose and extracellular metabolites

Determination of galactose and extracellular metabolites was performed as described (Asadollahi et al., 2008).

6.2.6 Extraction of carotenoids

The biomass from a certain volume of culture media was harvested by centrifuging at 5000 rpm for 10 min. The cell pellet was washed with distilled water and centrifuged for another 10 min at

5000 rpm. The cell pellet was transferred to a 2 ml FastPrep® tube and resuspended in 1 ml acetone containing 100 mg/L butylated hydroxytoluene (BHT). Then 200 µl of precooled glass beads (0.25-0.50 mm) were added to the tube. The cells were broken by vortexing in FastPrep® instrument (FP 120, BIO 101, Savant, Holbrook, NY) at the speed set to 6.5 m/sec for 4×45 sec separated by periods of cooling on ice.

6.2.7 Identification and quantification of carotenoids

In order to identify lycopene and phytoene, the acetone extract was dried in a vacuum centrifuge and resuspended in 300 µl acetonitrile. Lycopene and phytoene were identified by reverse-phase HPLC (Hewlett-Packard HP 1090 chromatograph series II with built in diode array detector and auto-injector). Analytes were separated on a Develosil column (C30-UG-5, Nomura Chemicals, Aichi, Japan) with a mobile phase consisting of acetonitrile and methanol. The analysis was performed at 40 ºC and it was monitored at 474 nm for lycopene and 286 nm for phytoene.

In order to quantify lycopene, the acetone extract was centrifuged at 20000 g for 5 min to remove the cell debris. The supernatant was filtered using 0.45 µm hydrophobic solvent resistant Minisart® filters (Sartorius AG, Göttingen, Germany). The OD of filtrate was measured at 474 nm. The amount of lycopene was determined using absolute standard curves obtained after each analysis run.

6.2.8 Fluorescence microscopy

Cells were grown in 5 ml of SC-his-ura liquid media containing 20 g/L galactose as carbon source. Cultures were grown on a shaker at 150 rpm at 25 ºC to allow the mRFP1 chromophore to form efficiently. At OD of 0.3 at 600 nm, cells from 1ml of culture were spun down, washed in SC-his-ura media and spun down again. Five microliters of cells were immobilized on a glass slide by mixing with a 37 ºC solution of 1.2% (w/v) low melting agarose (Nusieve 3:1 from FMC) containing the appropriate medium. Live cell images were captured with a cooled Evolution QEi monochrome digital camera (Media Cybernetics Inc., Bethesda, MD, USA) mounted on a Nikon Eclipse E1000 automated microscope (Nikon, Japan). Images were captured at 100-fold magnification using a Plan-Flour 100x 1.3 NA objective lens. The illumination source was a 103 W mercury arc lamp (Osram, Germany). The filter used to visualize crtI-mRFP1 (excitation 584 nm; emission 607 nm) was from Omega Optical (Brattleboro, VT, USA).

6.3 Results

6.3.1 Identification of lycopene formation in the engineered strains

The engineered strains harboring the genes for lycopene biosynthesis were grown in 5 L batch fermenters on galactose as the sole carbon source. The lycopene producing strains had a reddish color which was different from the control strains. This reddish color is an indication of lycopene accumulation in these strains but accumulation of lycopene was further confirmed by HPLC and comparing the peaks and UV spectra with those of standard lycopene.

6.3.2 Effect of *tHMG1* overexpression on lycopene accumulation

Overproduction of the catalytic domain of HMG-CoA reductase which catalyzes the major flux controlling step in the mevalonate pathway was concomitant with higher lycopene production. Overexpression of *tHMG1* from either the genome or a high copy number plasmid increased the lycopene accumulation. Overexpression of *tHMG1* from plasmid had a greater impact and the YIP-ML-06 strain yielded 0.51 mg/g DW lycopene. However, simultaneous overexpression of *tHMG1* from both plasmid and genome did not further improve lycopene production (Figure 6.1). These results are consistent with the earlier results on the effect of *tHMG1* overexpression on cubebol production (Chapter 4).

6.3.3 Effect of *ERG9* down-regulation on lycopene accumulation

Repression of *ERG9* reduces the carbon flux towards ergosterol and therefore more FPP will be available as a precursor for lycopene biosynthesis. In line with our previous results (Asadollahi et al., 2008; Chapter 4), squalene synthase down-regulation enhanced lycopene accumulation, but when it was combined with *tHMG1* overexpression, lycopene production was not further improved (Figure 6.1). Down-regulation of squalene synthase resulted in the formation of farnesol as an FPP derived by-product in sesquiterpene producing strains presumably as a consequence of poor expression of plant sesquiterpene synthase genes and accumulation of FPP (Asadollahi et al., 2008; Chapter 4). However, no accumulation of farnesol was observed for the YIP-ML-03 and YIP-ML-07 strains suggesting that optimizing codon usage led to diverting all the accumulated FPP towards carotenoids.

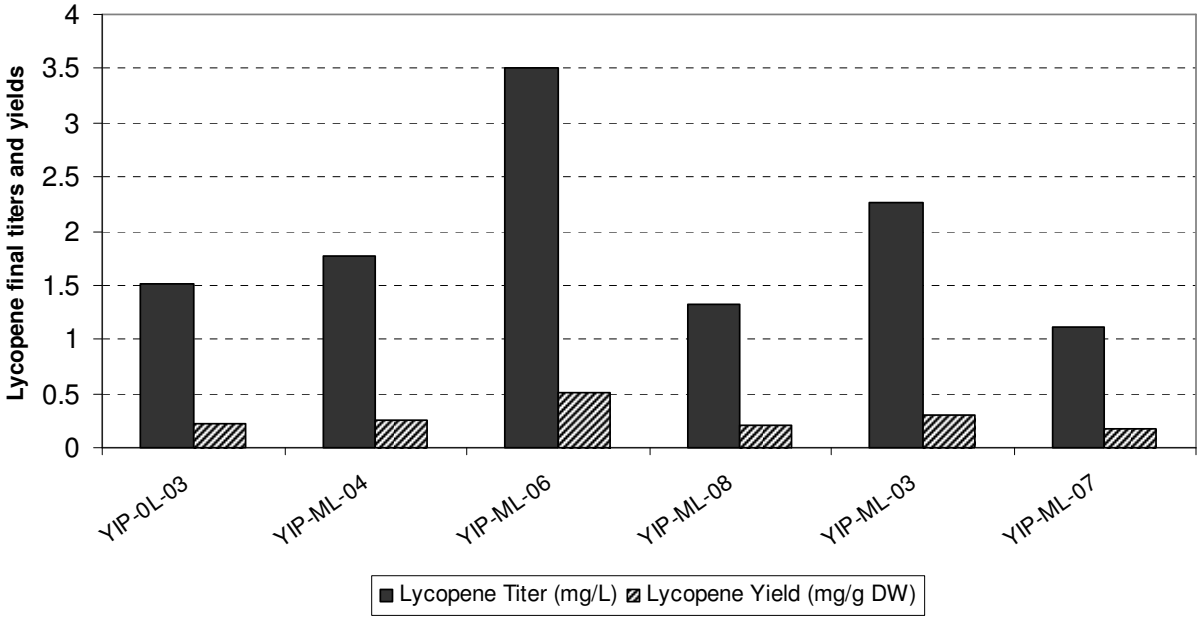

Figure 6.1. Final yields and titers of lycopene for the engineered yeast strains

6.3.4 Phytoene measurement

The last step in the lycopene biosynthetic pathway is conversion of phytoene, which is a colorless carotenoid to lycopene with a strong red color. This step is catalyzed by phytoene desaturase and accumulation of phytoene has been reported in the engineered carotenoid producing yeast strains (Yamano et al., 1994; Miura et al., 1998b; Verwaal et al., 2007). In order to investigate the formation of phytoene as a by-product in the lycopene producing yeast strains, first the peak for phytoene was identified for the phytoene producing yeast strains, YIP-0L-05 and YIP-ML-05. Phytoene formation for these two strains was confirmed by comparing the UV spectra of the phytoene putative peaks with the published UV spectra in the literature (Giuliano et al., 1986). The peak for phytoene was observed for both strains, but for the YIP-ML-05 strain an additional peak with an UV spectrum similar to that of phytoene was also observed which could be a phytoene derivative compound. The phytoene peak was also detected for the lycopene producing strains indicating that there is some flux control at the last step in the lycopene biosynthetic pathway. However, since we did not have access to commercial standard phytoene it was not possible to quantify phytoene level in the strains.

6.3.5 Localization of phytoene desaturase

Phytoene desaturase in the YIP-0L-06 strain was localized by tagging *crtI* with mRFP1 and examining the cells by fluorescence microscopy. YIP-0L-02 was used as a control strain in this experiment. Figure 6.2 shows bright field and fluorescence images for both YIP-0L-02 and YIP-0L-06 strains. As expected no signal was observed for the YIP-0L-02 strain (Figure 6.2-c), whereas a strong signal was observed for the mRFP1 protein in the YIP-0L-06 strain (Figure 6.2-d). Phytoene desaturase is believed to be a membrane integrated enzyme in natural carotenogenic organisms (Fraser et al., 1992). However, it does not seem to be an exclusively membrane associated enzyme in the YIP-0L-06 strain as the signal is not restricted to the membrane area.

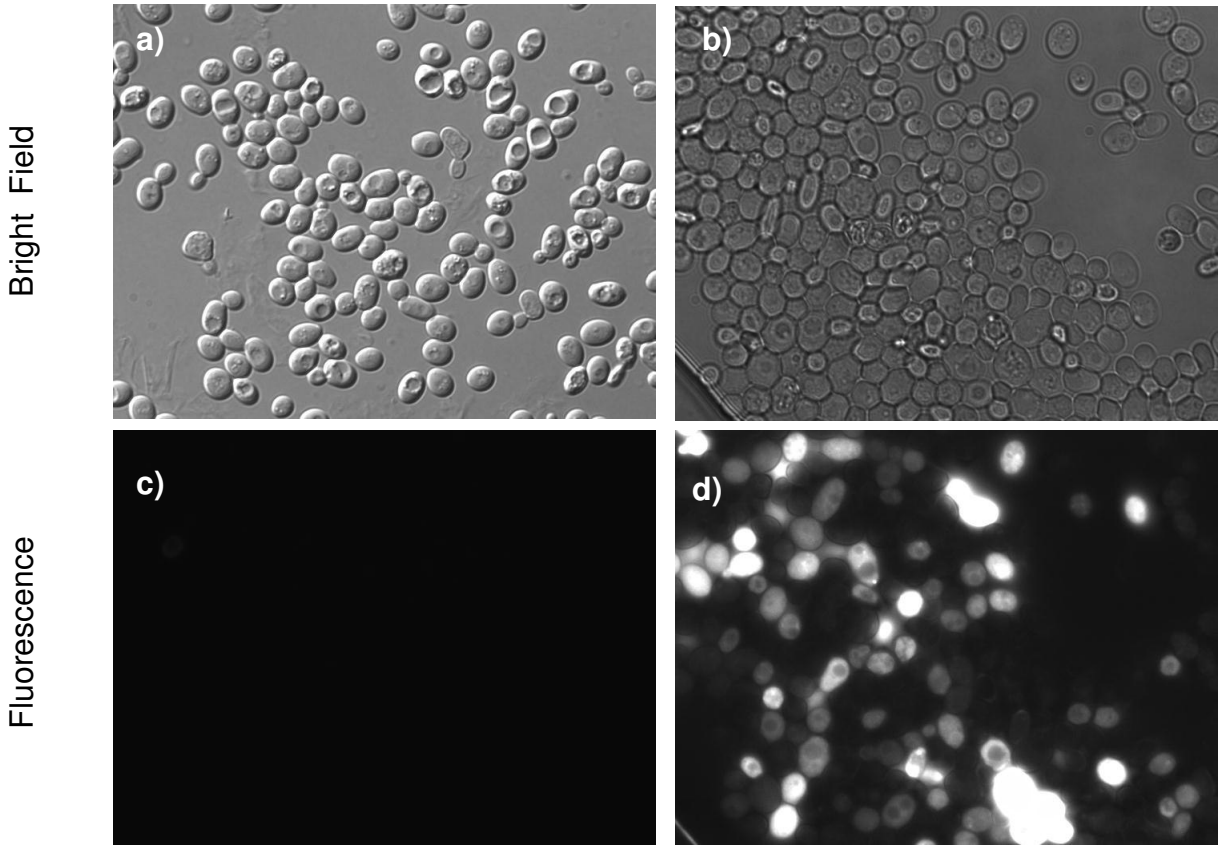

Figure 6.2. Microscopic images of yeast cells (a & b) Bright field images for the YIP-0L-02 (control) and YIP-0L-06 strains, respectively; (c & d) Fluorescence images of the YIP-0L-02 (control) and YIP-0L-06 strains, respectively

6.4 Discussion

The yeast *S. cerevisiae* was used as a platform for heterologous production of lycopene in this study. Expression of the *crtE*, *crtB*, and *crtI* genes allows biosynthesis of lycopene in yeast from FPP as precursor. Expression of codon optimized variants of amorphadiene synthase (Martin et al., 2003) and carotenogenic genes (Miura et al., 1998b) led to significant improvements in amorphadiene and carotenoid biosyntheses, respectively. Therefore the carotenogenic genes from *E. herbicola* were modified based on the optimal codon usage of yeast in the current study. Yeast cells transformed with the three crt genes accumulated 0.22 mg lycopene/g DW in a batch fermentation. Overproduction of HMG-CoA reductase which catalyzes a highly regulated step in the mevalonate pathway, by overexpression of *tHMG1* from either genome or a high copy number plasmid, increased the production of lycopene by 18% and 130%, respectively. Simultaneous overexpression of *tHMG1* from both the genome and plasmid did not further improve lycopene biosynthesis.

Earlier reports showed that down-regulation of squalene synthase encoded by *ERG9* resulted in a lower ergosterol content of yeast cells, increased sesquiterpene production and formation of farnesol as an FPP derived by-product (Asadollahi et al., 2008, Chapter 4). Repression of *ERG9* in the YIP-ML-03 strain increased lycopene production by 50% compared to the YIP-0L-03 strain. However, in line with the previous results (Chapter 4) lycopene production was not further improved by combining *ERG9* repression and *tHMG1* overexpression in the YIP-ML-07 strain.

Earlier we showed (Asadollahi et al., 2008; Chapter 4) substantially higher sesquiterpene titers compared to those obtained for lycopene in the same engineered yeast backgrounds in this study. For instance, an *ERG9* repressed yeast strain produced 35.3 mg/L total sesquiterpenes and 16.3 mg/L farnesol (Chapter 4), whereas the YIP-ML-03 strain in this study produced only 2.3 mg/L lycopene and did not produce any farnesol suggesting accumulation of other intermediates of the pathway. Accumulation of phytoene in the yeast strains engineered for carotenoid production (Yamano et al., 1994; Miura et al., 1998b; Verwaal et al., 2007) proposes that there is some flux control at the last step where phytoene is desaturated to form lycopene. We were also able to detect phytoene accumulation for the lycopene producing strains constructed here. Accumulation of phytoene is not due to restricted transcription of *crtI* as the expression level of *crtI* was found to be equal or even higher than that of other carotenogenic genes in the engineered yeast strains (Miura et al., 1998b; Verwaal et al., 2007). Since accumulation of phytoene has not been

observed in carotenoid production studies by *E. coli* the phytoene accumulation does not seem to be a consequence of a high K_m value of the phytoene desaturase. Conversion of phytoene to lycopene is accompanied with the removal of 8 hydrogen atoms from phytoene and formation of 4 additional conjugated double bonds leading to lycopene. There are contradictory reports on the cofactor requirement for this reaction in the literature (Fraser et al., 1992; Sandmann and Kowalczyk, 1989; Fraser and Bramley, 1994; Hausmann and Sandmann, 2000). Desaturation of phytoene in the daffodil chromoplast was shown to require molecular oxygen as the terminal electron acceptor (Beyer et al., 1989). However, the molecular oxygen can be replaced by quinones under anaerobic conditions (Mayer et al., 1990). In fact, quinones (plastoquinones in higher plants) function as intermediate electron carriers and are converted to hydroquinones (plastoquinol in higher plants). Plastoquinol can subsequently be reoxidized via photosynthetic electron transport chain or a tissue-specific redox component such as NADPH-dependent plastoquinone oxidoreductase in the tissues lacking photosynthetic electron transport chain (Norris et al., 1995). Functional expression of plant phytoene desaturase in *E. coli* (Bartley et al., 1999), which does not contain plastoquinone but only ubiquinone, implies that ubiquinones can take the same electron carrier role as plastoquinone. In prokaryotes the oxidative phosphorylation takes place in the inner cell membrane whereas in eukaryotes the protein complexes needed for the redox reactions are located in the mitochondria. Hence, it is likely that ubiquinone is more available for phytoene desaturase in bacteria than in yeast and this may explain why desaturation of phytoene functions more efficiently in bacteria.

In this study, we expressed the carotenogenic genes from a bacterium in a yeast strain. Therefore it is important to make sure that the phytoene desaturase has been localized properly. It was not possible to prove that phytoene desaturase is exclusively membrane integrated after examining yeast strain harboring *crtI* tagged with mRFP1 gene under fluorescence microscopy. Lang and coworkers (1994) overexpressed *crtI* from *Rhodobacter sphaeroides* in *E. coli,* but they found that phytoene desaturase is present in the cytoplasm of *E. coli*. They argued that this may be due to the fact that there is so high expression of phytoene desaturase that targeting to the membrane becomes saturated and therefore not all the enzyme becomes membrane associated. As the *crtI* gene used here has a bacterial origin it is possible that it is not targeted properly to the yeast membrane.

Consistent with our results, expression of carotenogenic genes from *E. uredovora* in *S. cerevisiae* also led to low levels of lycopene (Yamano et al., 1994). Recently, it was shown that transformation of *S. cerevisiae* with the carotenogenic genes from *Xanthophyllomyces dendrorhous* enables production of considerable amounts of carotenoids in *S. cerevisiae* but expression of the same genes in *E. coli* resulted in low carotenoid levels (Verwaal et al., 2007). Therefore, it seems that for heterologous production of carotenoids in *S. cerevisiae* it would be better to use carotenogenic genes from a yeast species such as *X. dendrorhous* than from a bacterium.

6.5 References

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Chapter 7

Conclusions and perspectives

Towards establishment of yeast as a platform for large scale production of sesquiterpenes the genes encoding three different sesquiterpene synthases namely valencene synthase, cubebol synthase and patchoulol synthase were expressed in *S. cerevisiae*. Expression of these genes resulted in accumulation of target sesquiterpenes in the medium culture. Valencene was the major product of valencene synthase but cubebol synthase and patchoulol synthase are multi-product enzymes and formed a range of sesquiterpenes with cubebol and patchoulol representing only 28% and 37% of the total sesquiterpenes, respectively.

Despite their high boiling points, the partial pressure of sesquiterpenes in the gas phase at low concentrations is very high presumably due to very large Henry's constants law of sesquiterpenes. This high partial pressure in the gas phase results in the loss of a significant amount of the excreted sesquiterpenes through the off-gas. Therefore the first part of the project aimed at developing an analytical platform for the measurement of total sesquiterpenes and subsequently characterization and comparison of sesquiterpene producing yeast strains (Chapter 2). A two-phase fermentation using an organic solvent such as dodecane as the secondary phase was shown to be an efficient system for trapping and quantification of total sesquiterpenes. This method was therefore used for the characterization of all sesquiterpene producing yeast strains throughout the project.

Expression of sesquiterpene synthase genes led to accumulation of low amounts of sesquiterpenes in a two-phase fermenter. The obtained titers were not sufficient for large scale production and therefore the rest of project was dedicated to design and use of metabolic engineering strategies to augment the intracellular pool of FPP. In the first attempt (Chapter 3), *ERG9* expression was attenuated by replacing the native *ERG9* promoter with a repressible *MET3* promoter in the presence of methionine. This resulted in reduced ergosterol content of yeast cells, increased production of sesquiterpenes and accumulation of farnesol as an FPP derived by-product. Accumulation of farnesol suggests that the sesquiterpene synthases are unable to divert all the available FPP towards the target sesquiterpenes and therefore FPP is converted to farnesol probably by the action of general phosphatases in the cell such as lipid phosphate phosphatase and diacylglycerol diphosphate phosphatase encoded by *LPP1* and *DPP1*, respectively.

HMG-CoA reductase catalyzes the main flux controlling step in the mevalonate pathway and overproduction of the catalytic domain of this enzyme by overexpression of *tHMG1* had a great impact on cubebol production. Overexpression of *tHMG1* also greatly impacted accumulation of squalene from trace amounts in the wild type and control strains to several milligrams per gram of biomass for the mutants (Chapter 4). This could be due to higher catalytic efficiency of squalene synthase compared to other enzymes at the FPP branch point of the mevalonate pathway including sesquiterpene synthases competing for FPP as substrate and therefore most of the FPP is converted to squalene. Surprisingly, squalene build up was also observed in the *ERG9* down-regulated strains if cubebol synthase was also expressed. Combination of *tHMG1* overexpression and *ERG9* down-regulation did not further improve cubebol production.

In silico metabolic engineering using the genome-scale reconstructed metabolic network of *S. cerevisiae* and MOMA approach revealed deletion of NADPH-dependent glutamate dehydrogenase encoded by *GDH1* as a new target gene for further improvement of sesquiterpene biosynthesis in yeast (Chapter 5). Deletion of *GDH1* enhanced cubebol titer by 85% but the cells had very low specific growth rates probably due to impaired ammonium assimilation. Overexpression of NADH-dependent glutamate dehydrogenase encoded by *GDH2* restored the specific growth rate to some extent.

The red color of lycopene can be used as a marker for high-throughput screening of lycopene overproducer mutants. The lycopene overproducers have most likely enhanced intracellular pools of isoprenoid precursors and therefore are probably able to overproduce other isoprenoids as

well. Motivated by this idea, in the last step of this project yeast strains capable of lycopene accumulation were constructed by expressing of *crtE*, *crtB*, and *crtI* genes from the bacterium *E. herbicola* in yeast (Chapter 6). In line with the results obtained in the previous chapters, both down-regulation of *ERG9* and overexpression of *tHMG1* led to enhanced accumulation of lycopene but combination of both modifications did not further improve lycopene biosynthesis. However the obtained lycopene titers were significantly lower than the sesquiterpene titers in the same background strains. Accumulation of phytoene and also low efficiency of bacterial carotenogenic genes in a yeast strain may explain the low lycopene titers.

One of the best engineered yeast strains in this study produced up to approximately 35.3 mg/L total sesquiterpenes, 16.3 mg/L farnesol and nearly 9 mg squalene/g DW. For a viable large scale production of sesquiterpene the total sesquiterpene titer should be increased further, and this may be accomplished by following some or all of the strategies mentioned below:

- All the characterization studies were carried out in batch fermenters in which the final biomass concentration was approximately 6-7 g DW/L. By running a fed-batch fermenter it is possible to increase the final biomass by almost 10-fold. This could lead to significantly higher titers of sesquiterpenes.
- A minimal medium was used in all fermentations. Optimization of medium composition can improve both specific growth rate and sesquiterpene biosynthesis significantly.
- Adjustment of methionine concentration in the culture medium of the *ERG9* down-regulated strains prevents relieved repression of *ERG9* during fermentation period and leads to higher sesquiterpene production as it was shown for patchoulol (Chapter 3).
- Accumulation of farnesol in the *ERG9* repressed strains may be suppressed by removing the phosphatase activities such as the ones encoded by *DPP1* and *LPP1*.
- Overexpression of *ERG20* could also have some enhancing effects on the sesquiterpene production.
- Combination of all the modifications including *ERG9* repression, *tHMG1* overexpression, *ERG20* overexpression, *DPP1* and *LPP1* deletion, *GDH1* deletion and *GDH2* overexpression in a single strain should improve sesquiterpene production.

- The sesquiterpene synthase genes used in this study were of a plant origin. Therefore optimization of the codon usage of the sesquiterpene synthases for yeast could be another strategy to enhance sesquiterpene production.
- Low catalytic efficiencies of the sesquiterpene synthases was probably one of the reasons for farnesol accumulation and squalene build up in the engineered yeast strains. It should be possible to improve the catalytic efficiency of these enzymes using protein engineering approaches. Although this needs an in-depth knowledge about the protein structure, active site and the mechanism of action of the enzyme this could remove one of the major bottlenecks in the pathway which is the synthase itself.
- More detailed experiments are required to gain more insights into the mechanism of squalene accumulation in the *ERG9* repressed strains in which cubebol synthase is also expressed. Understanding the mechanism and removing the cause is of crucial importance as it redirects substantial part of the flux from target sesquiterpenes to squalene which may have some cytotoxic effects.
- Heterologous expression of the MEP pathway in *S. cerevisiae* can be used as an alternative strategy to increase the flux towards isoprenoid precursors.
- High-throughput screening of the lycopene producing yeast mutants eased by the strong color of lycopene could be used as a metabolic engineering tool to find the lycopene overproducers that are most likely sesquiterpene overproducers as well. Libraries of lycopene overproducer yeast strains can be constructed using classical random mutagenesis, deletion libraries, transposon libraries etc. The overproducer mutants are then traced back to identify the genotypes causing the desired phenotype. This will subsequently lead to new ideas to augment intracellular pools of precursors. However, the phytoene accumulation needs to be circumvented before this strategy can be applied.

Appendix A

Sequences of natural and codon optimized *crtE*, *crtB*, and *crtI* genes from *E. herbicola*

crtE from *E. herbicola*

```
   1  ATGGTGAGTG GCAGTAAAGC GGGCGTTTCG CCTCATCGCG AAATAGAAGT
  51  AATGAGACAA TCCATTGACG ATCACCTGGC TGGCCTGTTA CCTGAAACCG
 101  ACAGCCAGGA TATCGTCAGC CTTGCGATGC GTGAAGGCGT CATGGCACCC
 151  GGTAAACGGA TCCGTCCGCT GCTGATGCTG CTGGCCGCCC GCGACCTCCG
 201  CTACCAGGGC AGTATGCCTA CGCTGCTCGA TCTCGCCTGC GCCGTTGAAC
 251  TGACCCATAC CGCGTCGCTG ATGCTCGACG ACATGCCCTG CATGGACAAC
 301  GCCGAGCTGC GCCGCGGTCA GCCCACTACC CACAAAAAAT TTGGTGAGAG
 351  CGTGGCGATC CTTGCCTCCG TTGGGCTGCT CTCTAAAGCC TTTGGTCTGA
 401  TCGCCGCCAC CGGCGATCTG CCGGGGGAGA GGCGTGCCCA GGCGGTCAAC
 451  GAGCTCTCTA CCGCCGTGGG CGTGCAGGGC CTGGTACTGG GGCAGTTTCG
 501  CGATCTTAAC GATGCCGCCC TCGACCGTAC CCCTGACGCT ATCCTCAGCA
 551  CCAACCACCT CAAGACCGGC ATTCTGTTCA GCGCGATGCT GCAGATCGTC
 601  GCCATTGCTT CCGCCTCGTC GCCGAGCACG CGAGAGACGC TGCACGCCTT
 651  CGCCCTCGAC TTCGGCCAGG CGTTTCAACT GCTGGACGAT CTGCGTGACG
 701  ATCACCCGGA AACCGGTAAA GATCGCAATA AGGACGCGGG AAAATCGACG
 751  CTGGTCAACC GGCTGGGCGC AGACGCGGCC CGGCAAAAGC TGCGCGAGCA
 801  TATTGATTCC GCCGACAAAC ACCTCACTTT TGCCTGTCCG CAGGGCGGCG
 851  CCATCCGACA GTTTATGCAT CTGTGGTTTG GCCATCACCT TGCCGACTGG
 901  TCACCGGTCA TGAAAATCGC CTGA
```

crtB from *E. herbicola*

```
   1  ATGAGCCAAC CGCCGCTGCT TGACCACGCC ACGCAGACCA TGGCCAACGG
  51  CTCGAAAAGT TTTGCCACCG CTGCGAAGCT GTTCGACCCG GCCACCCGCC
 101  GTAGCGTGCT GATGCTCTAC ACCTGGTGCC GCCACTGCGA TGACGTCATT
 151  GACGACCAGA CCCACGGCTT CGCCAGCGAG GCCGCGGCGG AGGAGGAGGC
 201  CACCCAGCGC CTGGCCCGGC TGCGCACGCT GACCCTGGCG GCGTTTGAAG
 251  GGGCCGAGAT GCAGGATCCG GCCTTCGCTG CCTTTCAGGA GGTGGCGCTG
 301  ACCCACGGTA TTACGCCCCG CATGGCGCTC GATCACCTCG ACGGCTTTGC
 351  GATGGACGTG GCTCAGACCC GCTATGTCAC CTTTGAGGAT ACGCTGCGCT
 401  ACTGCTATCA CGTGGCGGGC GTGGTGGGTC TGATGATGGC CAGGGTGATG
 451  GGCGTGCGGG ATGAGCGGGT GCTGGATCGC GCCTGCGATC TGGGGCTGGC
 501  CTTCCAGCTG ACGAATATCG CCCGGGATAT TATTGACGAT GCGGCTATTG
 551  ACCGCTGCTA TCTGCCCGCC GAGTGGCTGC AGGATGCCGG GCTGACCCCG
 601  GAGAACTATG CCGCGCGGGA GAATCGGGCC GCGCTGGCGC GGGTGGCGGA
 651  GCGGCTTATT GATGCCGCAG AGCCGTACTA CATCTCCTCC CAGGCCGGGC
 701  TACACGATCT GCCGCCGCGC TGCGCCTGGG CGATCGCCAC CGCCCGCAGC
 751  GTCTACCGGG AGATCGGTAT TAAGGTAAAA GCGGCGGGAG GCAGCGCCTG
 801  GGATCGCCGC CAGCACACCA GCAAAGGTGA AAAAATTGCC ATGCTGATGG
 851  CGGCACCGGG GCAGGTTATT CGGGCGAAGA CGACGAGGGT GACGCCGCGT
 901  CCGGCCGGTC TTTGGCAGCG TCCCGTTTAG
```

crtI from *E. herbicola*

```
   1  ATGAAAAAAA CCGTTGTGAT TGGCGCAGGC TTTGGTGGCC TGGCGCTGGC
  51  GATTCGCCTG CAGGCGGCAG GGATCCCAAC CGTACTGCTG GAGCAGCGGG
 101  ACAAGCCCGG CGGTCGGGCC TACGTCTGGC ATGACCAGGG CTTTACCTTT
 151  GACGCCGGGC CGACGGTGAT CACCGATCCT ACCGCGCTTG AGGCGCTGTT
 201  CACCCTGGCC GGCAGGCGCA TGGAGGATTA CGTCAGGCTG CTGCCGGTAA
 251  AACCCTTCTA CCGACTCTGC TGGGAGTCCG GGAAGACCCT CGACTATGCT
 301  AACGACAGCG CCGAGCTTGA GGCGCAGATT ACCCAGTTCA ACCCCCGCGA
 351  CGTCGAGGGC TACCGGCGCT TTCTGGCTTA CTCCCAGGCG GTATTCCAGG
 401  AGGGATATTT GCGCCTCGGC AGCGTGCCGT TCCTCTCTTT TCGCGACATG
 451  CTGCGCGCCG GGCCGCAGCT GCTTAAGCTC CAGGCGTGGC AGAGCGTCTA
 501  CCAGTCGGTT TCGCGCTTTA TTGAGGATGA GCATCTGCGG CAGGCCTTCT
 551  CGTTCCACTC CCTGCTGGTA GGCGGCAACC CCTTCACCAC CTCGTCCATC
 601  TACACCCTGA TCCACGCCCT TGAGCGGGAG TGGGGGGTCT GGTTCCCTGA
 651  GGGCGGCACC GGGGCGCTGG TGAACGGCAT GGTGAAGCTG TTTACCGATC
 701  TGGGCGGGGA GATCGAACTC AACGCCCGGG TCGAAGAGCT GGTGGTGGCC
 751  GATAACCGCG TAAGCCAGGT CCGGCTGGCG GATGGTCGGA TCTTTGACAC
 801  CGACGCCGTA GCCTCGAACG CTGACGTGGT GAACACCTAT AAAAAGCTGC
 851  TCGGCCACCA TCCGGTGGGG CAGAAGCGGG CGGCAGCGCT GGAGCGCAAG
 901  AGCATGAGCA ACTCGCTGTT TGTGCTCTAC TTCGGCCTGA ACCAGCCTCA
 951  TTCCCAGCTG GCGCACCATA CCATCTGTTT TGGTCCCCGC TACCGGGAGC
1001  TGATCGACGA GATCTTTACC GGCAGCGCGC TGGCGGATGA CTTCTCGCTC
1051  TACCTGCACT CGCCCTGCGT GACCGATCCC TCGCTCGCGC CTCCCGGCTG
1101  CGCCAGCTTC TACGTGCTGG CCCCGGTGCC GCATCTTGGC AACGCGCCGC
1151  TGGACTGGGC GCAGGAGGGG CCGAAGCTGC GCGACCGCAT CTTTGACTAC
1201  CTTGAAGAGC GCTATATGCC CGGCCTGCGT AGCCAGCTGG TGACCCAGCG
1251  GATCTTTACC CCGGCAGACT TCCACGACAC GCTGGATGCG CATCTGGGAT
1301  CGGCCTTCTC CATCGAGCCG CTGCTGACCC AAAGCGCCTG GTTCCGCCCG
1351  CACAACCGCG ACAGCGACAT TGCCAACCTC TACCTGGTGG GCGCAGGTAC
1401  TCACCCTGGG GCGGGCATTC CTGGCGTAGT GGCCTCGGCG AAAGCCACCG
1451  CCAGCCTGAT GATTGAGGAT CTGCAATGA
```

Sequences of synthetic crt genes

crtE codon optimized

```
   1  ATGGTTTCTG GTTCGAAAGC AGGAGTATCA CCTCATAGGG AAATCGAAGT
  51  CATGAGACAG TCCATTGATG ACCACTTAGC AGGATTGTTG CCAGAAACAG
 101  ATTCCCAGGA TATCGTTAGC CTTGCTATGA GAGAAGGTGT TATGGCACCT
 151  GGTAAACGTA TCAGACCTTT GCTGATGTTA CTTGCTGCAA GAGACCTGAG
 201  ATATCAGGGT TCTATGCCTA CACTACTGGA TCTAGCTTGT GCTGTTGAAC
 251  TGACACATAC TGCTTCCTTG ATGCTGGATG ACATGCCTTG TATGGACAAT
 301  GCGGAACTTA GAAGAGGTCA ACCAACAACC CACAAGAAAT TCGGAGAATC
 351  TGTTGCCATT TTGGCTTCTG TAGGTCTGTT GTCGAAAGCT TTTGGCTTGA
 401  TTGCTGCAAC TGGTGATCTT CCAGGTGAAA GGAGAGCACA AGCTGTAAAC
 451  GAGCTATCTA CTGCAGTTGG TGTTCAAGGT CTAGTCTTAG GACAGTTCAG
 501  AGATTTGAAT GACGCAGCTT TGGACAGAAC TCCTGATGCT ATCCTGTCTA
 551  CGAACCATCT GAAGACTGGC ATCTTGTTCT CAGCTATGTT GCAAATCGTA
 601  GCCATTGCTT CTGCTTCTTC ACCATCTACT AGGGAAACGT TACACGCATT
 651  CGCATTGGAC TTTGGTCAAG CCTTTCAACT GCTAGACGAT TTGAGGGATG
 701  ATCATCCAGA GACAGGTAAA GACCGTAACA AAGACGCTGG TAAAAGCACT
 751  CTAGTCAACA GATTGGGTGC TGATGCAGCT AGACAGAAAC TGAGAGAGCA
 801  CATTGACTCT GCTGACAAAC ACCTGACATT TGCATGTCCA CAAGGAGGTG
 851  CTATAAGGCA GTTTATGCAC CTATGGTTTG GACACCATCT TGCTGATTGG
 901  TCTCCAGTGA TGAAGATCGC CTAA
```

crtB codon optimized

```
   1  ATGAGTCAAC CACCTTTGTT GGATCATGCT ACTCAAACGA TGGCTAATGG
  51  TTCCAAGTCC TTTGCTACAG CAGCTAAACT GTTTGACCCA GCTACTAGAA
 101  GATCAGTGCT TATGCTGTAC ACTTGGTGTA GACACTGTGA TGACGTTATA
 151  GATGACCAGA CACATGGTTT CGCATCTGAA GCTGCTGCAG AAGAAGAGGC
 201  TACTCAGAGA TTGGCTAGAT TGAGAACGCT TACACTTGCA GCTTTTGAAG
 251  GTGCTGAGAT GCAAGATCCT GCTTTTGCTG CATTCCAAGA AGTTGCACTA
 301  ACACACGGTA TTACGCCAAG AATGGCACTT GATCACTTGG ATGGTTTCGC
 351  AATGGATGTT GCTCAAACTC GTTACGTGAC CTTTGAAGAC ACCTTGAGAT
 401  ACTGCTACCA TGTTGCTGGA GTAGTTGGTT TGATGATGGC AAGAGTAATG
 451  GGTGTAAGAG ACGAAAGGGT TTTGGACAGA GCTTGTGATC TAGGTTTGGC
 501  TTTTCAGCTG ACAAACATCG CGAGAGATAT TATCGACGAT GCAGCTATTG
 551  ACAGATGCTA TCTACCTGCT GAATGGTTGC AAGATGCTGG TCTAACTCCT
 601  GAGAATTACG CTGCAAGAGA GAACAGAGCT GCATTAGCAA GAGTTGCTGA
 651  AAGGCTGATA GACGCTGCTG AACCCTATTA CATCTCAAGT CAAGCTGGAT
 701  TGCATGATCT ACCACCTAGA TGTGCTTGGG CTATAGCTAC TGCAAGATCT
 751  GTCTACAGAG AGATTGGCAT CAAGGTAAAA GCTGCAGGTG GTTCTGCTTG
 801  GGATAGACGT CAACACACTA GCAAAGGAGA GAAGATTGCG ATGCTTATGG
 851  CTGCACCAGG ACAAGTCATT CGTGCCAAAA CAACCAGAGT TACACCAAGA
 901  CCTGCTGGTT TATGGCAAAG ACCTGTCTAA
```

crtI codon optimized

```
   1  ATGAAGAAAA CCGTAGTGAT TGGTGCAGGT TTTGGTGGTT TAGCTTTGGC
  51  TATACGTCTA CAAGCTGCAG GTATTCCTAC AGTGCTATTG GAGCAAAGAG
 101  ACAAACCAGG AGGAAGAGCT TATGTTTGGC ACGATCAAGG CTTTACCTTT
 151  GATGCTGGTC CTACAGTCAT CACTGATCCT ACTGCATTGG AAGCTTTGTT
 201  CACCTTAGCT GGTAGAAGAA TGGAAGATTA TGTCCGTCTA TTGCCTGTCA
 251  AGCCGTTTTA CAGATTGTGT TGGGAATCTG GTAAAACCCT AGATTACGCC
 301  AATGACAGTG CTGAACTAGA AGCTCAGATT ACGCAGTTTA ATCCCAGAGA
 351  TGTCGAAGGT TACAGGAGAT TCCTTGCCTA TTCCCAAGCT GTTTTCCAAG
 401  AGGGTTATCT TCGTTTGGGT TCAGTTCCAT TCCTGTCCTT TAGGGATATG
 451  CTTAGAGCAG GTCCTCAGTT GTTGAAGCTA CAAGCATGGC AAAGTGTGTA
 501  TCAGTCTGTT TCGAGATTTA TCGAGGATGA ACATCTGAGA CAAGCATTCT
 551  CATTCCACAG TCTTCTAGTT GGAGGTAATC CCTTTACCAC ATCGAGCATA
 601  TATACGTTGA TTCACGCTTT GGAAAGAGAA TGGGGAGTTT GGTTTCCTGA
 651  AGGTGGAACA GGTGCTTTGG TTAATGGTAT GGTGAAGCTA TTCACGGATT
 701  TGGGTGGAGA AATAGAGCTG AATGCAAGAG TGGAAGAACT TGTTGTAGCA
 751  GACAACAGAG TCTCACAAGT TAGACTTGCT GATGGTAGGA TCTTCGATAC
 801  AGATGCTGTA GCTTCAAACG CAGATGTAGT GAACACTTAT AAAAAGTTGT
 851  TGGGACATCA TCCTGTTGGA CAAAAGAGAG CAGCTGCTTT GGAGAGGAAA
 901  TCTATGAGCA ACTCGTTGTT TGTCCTTTAC TTTGGGCTGA ATCAACCACA
 951  CTCACAACTA GCTCATCACA CAATCTGCTT TGGTCCTAGA TACAGAGAGC
1001  TGATAGATGA AATTTTCACT GGATCTGCTT TAGCAGACGA TTTTTCCCTG
1051  TACTTGCATT CACCATGTGT TACTGATCCC TCTTTAGCAC CACCTGGTTG
1101  TGCTAGCTTC TATGTACTAG CACCTGTACC ACATTTGGGT AATGCTCCAT
1151  TAGATTGGGC ACAAGAAGGA CCGAAATTGA GGGATAGGAT CTTCGACTAT
1201  TTGGAAGAAC GTTACATGCC AGGTTTGAGA TCTCAGTTGG TTACACAGAG
1251  GATATTCACA CCAGCTGATT TTCATGATAC TCTAGATGCG CATTTAGGTA
1301  GCGCTTTTTC CATTGAGCCA CTTTTGACGC AAAGTGCTTG GTTTAGACCA
1351  CACAACAGAG ATTCTGACAT TGCCAATCTG TACCTAGTAG GTGCAGGAAC
1401  TCATCCAGGA GCTGGTATTC CTGGAGTTGT AGCTTCTGCT AAAGCTACTG
1451  CTAGTCTGAT GATCGAGGAT TTGCAGTAA
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